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THE RELATIONSHIP BETWEEN THE THYMUS AND REPRODUCTION

DOROTHY H. ANDERSEN

Department of Pathology, College of Physicians and Surgeons, Columbia University, New York City

The earlier observers of the thymus gland were impressed with the fact that in the newborn it is a large organ, while in the adult it is small and sometimes difficult to find. The natural conclusion was that the thymus was a gland somehow related to growth and of no use in later life. The long series of attempts to remove the gland, beginning with Restelli in 1845 and Friedleben in 1858, were made for the most part with the expectation of producing some abnormality in the growth or development of the animal, especially of the skeleton. It was at least demonstrated that the thymus was not essential to life. Beyond this the results were various. The most striking were those of Basch (1906), Klose and Vogt (1910), and Matti (1912), which seemed to establish a definite relationship between thymectomy and the abnormal development of the bones. Others, as Hammar (1905) in frogs, Paton and Goodall (1904) in guinea pigs, Pappenheimer (1914) in rats, Marine and Manley (1917) in rabbits, and Park and McClure (1919) in dogs, could demonstrate no abnormalities after thymectomy in young animals. The analytical review of Park and McClure convinced experimenters that the bone changes found by some of the workers were due to intercurrent rickets, and that thymectomy did not materially alter the general development of the animal.

The hypothesis of a physiological relationship between the thymus and the gonads was first put on an experimental basis by Calzolari (1898). He castrated 6 young male rabbits, keeping controls of the same age. Sometime later he autopsied them and found that the thymus was heavier in the castrated animals than in the controls.

During the present century a great number of investigators have carried out experiments in the faith that the thymic-gonadal relationship is an intimate one. Much of the work is without value because of the great individual variation in normal animals. Moreover, the involution of the thymus due to malnutrition and to intercurrent infection was disregarded, and the resulting necessity for a long series of well-controlled animals to establish a point was not clearly seen. The present review is an attempt to evaluate the evidence offered as to the relationship between the thymus and the sex glands.

The efforts to demonstrate a thymic-gonadal relationship have been made along the following lines of investigation: 1, the weight of the thymus as affected by various phases of normal sex life, as puberty, mating, pregnancy and lactation; 2, the effect of the removal of the gonads on the thymus; 3, the effect of thymectomy on sexual anatomy and physiology; 4, the effect upon the thymus of the administration of gonadal substance by feeding, injection or transplantation; 5, the effect of the administration of thymus substance upon the sexual apparatus. The data which have been offered are discussed under these headings.

Thymus weight during various phases of sex life. The most comprehensive study of the thymus gland up to the present day is that of Friedleben in 1858. Besides careful anatomical studies in various vertebrates and a number of extirpation experiments Friedleben reported a series of 320 human cases, with the weight of the thymus, the age and the cause of death. He concluded that the thymus was heaviest soon after birth and decreased steadily in weight thereafter. Perusal of his protocols shows that many of the infants died at birth of asphyxia and were well-nourished, while the children and young adults for the most part died of acute infections. We now know that acute infections are a cause of rapid "accidental involution," but that well-nourished individuals dying suddenly have large thymuses, so that his conclusions were made on unsound evidence.

The next important contribution to our knowledge of thymus weight in relation to age is that of Hammar (1906) who gave a complete review of the earlier literature as a preface to his careful analysis of 123 cases from human autopsy material. Hammar showed clearly that the thymus was on the average heavier during the 11–15 year age period, with a steady increase in weight in the 1–5 and 6–10 year periods and a decrease during the 16–20 year and later periods. However, the relative weight of the thymus in terms of grams per kilo of body weight was greatest in the newborn, with a gradual decrease thereafter. The

individual variation was great, and he considered that the heavier average weight in male over female thymuses after the age of sixteen was not significant for this reason. Hammar introduced the measurement of the various components of the gland, the medulla, cortex and connective tissue, and he also counted the Hassall corpuscles. The fact that human material must always be pathological makes for greater variation in total thymus weight as well as in the proportions of cortex and medulla. Acute infections (Hammar, 1927) and starvation (McCarrison, 1921) both make for rapid loss of thymic tissue at any age. The chief implication in regard to the physiology of the thymus from Hammar's work was that since the weight of the organ increased before puberty, there must be some relationship between the thymus gland and the maturing sexual apparatus.

Hatai (1914) has confirmed Hammar's observations in the rat, showing a sharp rise in thymus weight to a peak at 85 days with a more gradual decrease thereafter. This is a little later than puberty, which occurs at 72 days in the female rat when the criterion is the opening of the vagina. Hatai's animals were normal and the series is the longest one which has been used to prove this point. The same author (1913) showed a sharp increase in the weight of the testes between 50–80 days and in the ovaries between 60–110 days of age. Similar studies on the guinea pig by Paton and Goodall (1904) showed that the actual weight of the thymus was greatest when the guinea pig weighed 200–300 grams, which is the period of puberty in this animal; the greatest relative weight was found at 350 grams, or at about 2–3 months of age.

Henderson (1904) found a decrease in thymus weight in heifers after the age of 15 months and in bulls after the age of 21 months. Gedda (1921) reported work on rabbits in which he found that after the sharp post-pubertal decrease in thymus weight there was a gradual rise to about the level that obtained at puberty. Careful study of the gland showed that this second increase of weight was due to an increase in the interstitial tissue, while the weight of the cortex and medulla decreased steadily. Hammett (1926) found a similar secondary increase in thymus weight in rats but did not section the glands, and assumed that the increase of weight was due to increase of parenchyma. This error was pointed out by Hammar (1927). Blom and Aderman (1922) reported similar findings in the dog, and noted that the average number of Hassall's corpuscles decreased with age.

In 1930 Kinugasa undertook a similar study in the rat, apparently without being aware of Gedda's work. He studied the thymus in 66

rats of various ages between 1 and 240 days, of which all but 4 were 120 days or under. He weighed the glands and showed that those from the 8 rats killed at the age of 70 days weighed on the average more than the ones from rats killed at 60 and 90 days of age. He gave the age of puberty as 60 days and concluded that the thymus weighed the most on about the tenth day after puberty. He also made serial sections of these glands and calculated the relative volume of cortex and medulla. He found the cortex at birth to form 70 per cent of the gland and at 7 days, 85 per cent. After this time the relative volume of the cortex gradually decreased to 75 per cent at 60 days of age and to 70 per cent in rats over 120 days. He drew the conclusion from his abundant data that the cortex of the thymus and the gonads acted antagonistically to each other's development, while the medulla and the gonads acted synergistically. The remarkable uniformity of results in these and other experiments of his, in contrast to the great variability of thymus weight and structure as found by other workers, cast some suspicion on the validity of his findings. Riddle and Frey (1925) after observing the glands in 903 pigeons of several breeds stated that the relative and absolute weights of the thymus were greatest in birds 3 months after the egg containing them was laid, that is, about 10 weeks after hatching. These birds are sexually mature at 5–8 months, usually at 6 months of age. The greatest thymus weight therefore preceded the laying of the first egg by about 2 months. Data on the lower vertebrates are wanting.

In the experiments quoted above there is a difference of opinion as to whether the maximum weight of the thymus precedes, accompanies, or follows the onset of puberty. No experiments have been found in which the sexual organs of the animals used were studied with sufficient care to establish this point conclusively, but the more careful work seems to indicate that the maximum weight is found a little after the onset of puberty.

There are histological differences between the thymi of age involution and of accidental involution following infection. In the former there is a decrease of the cortex and medulla, and of the number of Hassall's corpuscles, with an increase of the interstitial tissue (Gedda, 1921; Blom and Aderman, 1922). In acute infections there is a loss of parenchyma, the interstitial tissue is not significantly changed and there is an increase in the number of Hassall's corpuscles (Hammar, 1918, 1923, 1927).

A sex difference does not appear striking in the longer series. Ham-

mar (1906) reported no difference in man up to the age of 16 years, after which the male thymus was heavier, but he added that in view of the great variability of thymus weight his series was not long enough to establish the point. The same criticism can be made of Paton's finding (1911) that the female guinea pig has the larger thymus. Squadrini (1909) found that age involution occurred later in cows than in bulls. Riddle and Frey (1925) reported that in immature pigeons the female thymus had a larger relative weight than the male thymus, but the same actual weight, while after puberty the male thymus was slightly heavier. Inspection of their data, however, leaves us convinced that these findings are probably due to the usual variability. It may be concluded that there is but little evidence for any sex difference in thymus weight.

In regard to the effect of the mating of animals on the weight of the thymus, there are notes by Henderson (1904) and by Knipping and Rieder (1924). Henderson stated that in his series the young bulls which had been used for serving cows showed more marked atrophy of the thymus than *virgin* animals of the same age. The difference was definite in the bulls under 2 years of age, of which his series contained 12 mated and 8 unmated animals. In the older bulls the difference was less marked.

Knipping and Rieder compared the thymuses of mated guinea pigs with virgin controls of the same age. They used 20 guinea pigs but did not state how many were controls and gave no weights. The animals were all killed at the age of 13 weeks. They found that the weight of the thymus was 27 per cent greater in the unmated controls than in the mated animals. Since the sex was not noted and the thymus was removed by operation with no general autopsy, we are left in doubt as to whether some were not pregnant females which would have small thymuses. The microscopic picture was that of age involution. The evidence of Henderson is much more convincing, although it does not appear invincible. Since in his series there was very little difference between the mated and virgin animals over 2 years, we can only say that possibly early mating hastens the involution of the thymus.

Involution of the thymus during pregnancy has been noted by many workers, but each has reported only a few cases. No single study with a complete protocol and adequate controls has been found. The most complete is that of Jolly and Lieure (1930) on 20 guinea pigs (number of controls not stated). These workers mated young animals and kept the litter-mate controls with the mother. They stated that with four

exceptions the thymi of the pregnant animals weighed one-third to one-half times as much as did those of the controls. No actual weights and no protocols were given. Henderson (1904) observed that the thymus was atrophied in two pregnant heifers. In Hammar's series (1906) there were two pregnant women whose glands were small. Ronconi (1909) reported one case in a woman. Squadrini (1909) stated that repeated pregnancies favor thymic involution in the cow. Winiwarter (1926) observed that the islands of thymic tissue usually present in the cat thyroid were involuted or missing in the pregnant cat. The longest and most convincing series is that of Hammar (1926) who collected 24 cases of women dying suddenly of accidental causes during pregnancy. In these cases the thymus gland was on the average slightly above the lower limit of the normal range. It is impossible to be sure of the point without well controlled work on animals, but it is easy to believe that the increased burden of pregnancy especially during the latter part of it, might decrease the weight of the thymus.

The possibility of a post-partum restoration of the thymus was suggested by Fulci (1913) who excised portions of the thymus during the latter days of pregnancy in rabbits and compared the microscopic structure with that of the remainder of the gland which was obtained at autopsy at various intervals after parturition. He apparently removed the young after parturition, and concluded that the thymus regenerated during the 2–3 weeks after pregnancy. The number of animals used is not given. Bompiani (1914) confirmed Fulci's findings. He also reported that the involution of the thymus during pregnancy continued throughout lactation. He killed lactating rabbits at 15, 28 and 40 days post-partum. As controls he used animals whose young had been removed at birth and who were killed at various intervals. A second set of controls were rabbits that were allowed to nurse their young 15 or 20 days, were then weaned, and killed at various periods after weaning. The age of the animals, the number of animals, the actual weights of the thymi and the differences in weight are not given. He also found that lactation delayed the appearance of the post-partum regeneration of the thymus. Jolly and Lieure (1930) also corroborated Fulci's findings. They killed one group of rabbits at parturition, another group 3 weeks after, and, as controls, a third group of virgin rabbits of the same age. They also found post-partum regeneration of the thymus "within physiological limits."

In view of the intimate connection between the general state of nutrition and thymus weight it seems likely that the thymus does lose

weight during pregnancy and lactation, but this concept and the less probable one of regeneration after pregnancy are based on very few animals. To be convincing, experiments of this type should be done on very young animals of known age and weight, in their first pregnancy, and there should be a sufficient series with controls to allow of statistical analysis of the actual and relative weight of the thymus in the various conditions.

The effect of the extirpation of testes and ovaries on the thymus. The first attempt to solve the thymus problem by the method of castration was made by Calzolari, who found that 6 rabbits, castrated when young and examined some weeks later, had larger thymus glands than their 6 controls of the same age. In 1904 Henderson, observing slaughter-house material, stated that butchers had long known that the thymus was larger in oxen than in bulls and reported weights on a series of 52 oxen, 32 bulls and 30 cows of various ages up to 4 years. The oxen were usually castrated when 1–3 months of age. In his series the thymus was the same weight in bulls and heifers of corresponding ages, but was about twice as heavy in the oxen, especially in the older ones.

Three more items of information on the point have been added. First, the figures of Henderson (1904) and Squadrini (1909) showed that the difference between the size of the thymus in castrated and in control animals was not marked until puberty, and that with the onset of age involution in the controls this difference increased. The more recent short series of rabbits of Jolly and Lieure (1929) contributed evidence in the same direction. The second point bears upon the effect of castration upon the thymus in mature animals. Five young adult guinea pigs castrated by Halnan and Marshall (1914) and killed a few weeks later showed thymus glands which were on the average about 50 per cent heavier than their 5 controls; 7 guinea pigs castrated before puberty and autopsied after maturity had thymus glands twice as heavy as those in the 10 controls. No experiment in aged animals has come to light. The third point in regard to the large thymus in the castrated animals was brought out by Squadrini (1909) who showed, in a slaughter-house series of oxen and bulls, that although the ox thymus was larger than the bull thymus, it was smaller in the 12 year old oxen than in the 5 year old ones, and that the involution was delayed, but not prevented by castration. This was confirmed by Marine, Manley and Baumann in 1924.

Some of the workers who have given experimental evidence of delayed involution of the thymus following castration, besides those already

mentioned, are Goodall (1905) in the guinea pig; Soli (1907, 1909) in the chicken and rabbit; Marassini (1910) in the sheep, cow and guinea pig; and Paton (1911) in the guinea pig. No discordant note has been found.

The evidence concerning the effect of spaying the female is less complete, but tends in the same direction. The earliest experiments that have come to notice are those of Soli (1909) who spayed 3 rabbits and stated that the thymus in these animals was heavier than in the 3 controls of the same age; and of Valtorta (1909) who found the same thing in 6 rabbits but mentioned no controls. Marassini (1910) in 6 guinea pigs, with controls but without a complete protocol, reported the same finding; so also did Halnan and Marshall (1914). Paton (1911) reported no difference between 4 experimental animals and 7 controls. In all this work on the effect of castration and spaying on the age involution of the thymus there is no single well-controlled series of adequate length. The evidence, with the exception of Paton's 4 guinea pigs, is uniformly in support of the original conclusion of Calzolari; namely, that removal of the gonads in the young animal delays the age involution of the thymus.

There have been several attempts at extirpation of both the thymus and the gonads in the same animal. Paton (1911) reported that the double operation had no effect on the female, but checked growth in the male. Halnan and Marshall performed the double operation on 12 males with 13 controls and found no delay in growth. Some retardation might be expected from the trauma of two severe operations in the young animal.

The delayed involution of the thymus as a result of removal of the gonads in the young animals appears so well established that no further effort seems necessary to confirm it.

The effect of thymectomy on the sex glands. In view of the frequently confirmed finding of delayed involution following early castration, it has seemed possible to many people that thymectomy might have some direct effect on the testes and ovaries. The prolific earlier workers on thymectomy experiments were absorbed in getting their animals to survive the operation, and later, in the bone lesions of the surviving animals. Most of the animals died early with cachexia, and the underdevelopment of the gonads was plausibly explained on this basis by most of the workers. The first investigator to report positive findings of gonadal changes following thymectomy was Paton (1905). He operated upon 24 guinea pigs between the ages of 1 and 45 days, having 23

controls, killed the animals at a weight of 115–355 grams and weighed the testes and epididymi. He reported that in the guinea pigs killed below the weight of 200 grams the testes were 27 per cent heavier in the operated than in the controls on the average, while in the larger animals there was no significant difference. Upon this evidence he postulated a gonad-inhibiting function for the thymus. Soli (1907) at first corroborated this on a small series of cocks. In 1909 he reported a longer series of guinea pigs and cocks. In the guinea pigs he found no difference in the weight of the testes in the thymectomized and control animals but in the cocks he found the testes of the controls heavier, with the exception of two operated pairs which were killed in the spring when the testes are usually heavier. A careful survey of both protocols reveals the usual wide variation in thymus weight which is so great as to make the difference between the operated and control animals insignificant. Halnan and Marshall (1914), using a long series of guinea pigs, were unable to confirm Paton's results and found no significant difference in the weight of the testes between the operated and control animals. At their request Yule (1914) analysed Paton's figures statistically and found the probable error to be so great as to obscure any differences between the operated and control testes. In 1911 Paton repeated his experiments and confirmed his original findings. Meanwhile other workers reported the precocious appearance of puberty following thymectomy. Klose and Vogt (1910) recorded a transient hyperplasia of the testes which disappeared when their dogs became cachectic. Lampé (1912), giving no protocol, said that after thymectomy in the dog, the sex glands weighed two to three times as much as those of the control animals. Adler (1914) reported longer gonads in the three completely thymectomized frogs than in the remaining 17 incompletely extirpated animals of the 20 survivors out of 950 operated animals. As Allen (1920) pointed out, Adler neglected to state the sex of his animals, and the female gonad is normally much longer than the male. Allen himself in 14 tadpoles thymectomized at 8 to 8.5 mm. obtained metamorphosis and gonads of the same size as the 36 control animals.

Among those reporting a decrease in the size of the gonads following thymectomy are Valtorta (1909) in female rabbits; Soli (1909) in female guinea pigs; Hart and Nordmann (1910) in both male and female dogs; Lucien and Parisot (1910), who reported transient retardation of the development of the testes in male rabbits; and Pighini (1922) in the chick (both male and female). In all of these experiments the general development of the animal was poor, and several of the workers, as

Valtorta and Pighini, interpreted the under-development of the gonads to these obvious causes.

The experimenters reporting no change after thymectomy are more numerous. Halnan and Marshall and Allen have already been mentioned. Bracci (1905) found no changes in the testes of thymectomized rabbits on histological examination, as compared with litter-mate controls, but neglected to state the number and ages of his animals. Ranzi and Tandler (1909) in the guinea pig and Matti (1911) in the dog reported negative findings but neither made a complete study of the organs. Soli (1909) in a series of male guinea pigs killed at 480 and 680 grams found no changes. Renton (1916) thymectomized 6 guinea pigs at 15 to 20 days with negative results. Pappenheimer (1914), working on rats, found no changes. Park and McClure (1919), on dogs, found no differences in the gonads following thymectomy with the exception of two operated dogs which had also had distemper. Ackert and Morris (1929) and Morgan and Grierson (1930) reported negative results in chickens. Walter (1930) operated on 10 rats, aged 4 to 5 weeks and weighing 20 to 24 grams, all of which died within 12 days after the operation; there were no significant changes in the gonads. Of these experiments, the ones which were best controlled were those of Halnan and Marshall, Allen, Soli, Pappenheimer, and Morgan and Grierson. The work of Pappenheimer is especially valuable because the thymectomy was proven by serial section to be complete, the animals were in good health and the testes were examined histologically. This series is too small to cast light on the question as to whether thymectomy hastens, delays, or does nothing to the age of puberty, but his experiments prove conclusively that spermatogenesis takes place normally after complete extirpation of the thymus.

The longest series of animals, reported by Andersen (1931), includes 86 thymectomized female rats with 85 controls from the same litters and 40 thymectomized male rats with 41 controls from the same litters. The diet and living conditions were standardized. The opening of the vagina was used as the criterion for puberty in the female animals, as this occurs at the first estrous period in half the cases and precedes the first estrous period by only a few days in the remaining ones. The experiment was divided into three parts. Series I, consisting of 26 operated and 26 control rats, which were born in the winter and operated on during the third or fourth week, reached puberty at the mean age of 58.8 $\pm$2.5 days for the operated rats and 58.6 $\pm$1.9 for the controls. The mean weight at the onset of puberty was 96.0 $\pm$1.9 grams in the

operated rats and 102.0 ±2.1 in the controls. Series II, consisting of 39 operated and 40 control rats, born in the spring and operated upon on the 21st day of age reached puberty at the mean age of 49.4 ±0.8 days (operated) 47.8 ±0.7 days (controls) and the mean weight of 93.6 ±1.6 grams (operated) and 90.8 ±1.5 grams (controls). Series III, born during the winter and spring and thymectomized at the age of one day, reached puberty at the mean age of 47.5 ±1.5 grams (operated) and 46.7 ±1.4 days (controls) and the mean weight of 93.3 ±1.5 grams (operated) and 92.1 ±1.5 grams (controls).

The criterion of puberty in the male rats was the presence of masses of mature motile sperm in the tail of the epididymis. This was first found at the age of about 60 days, and in all cases before 70 days. There was no difference between the operated and control animals. These experiments, which were controlled as to diet, living conditions, season, and completeness of the thymectomy, demonstrate convincingly that thymectomy does not affect the age of puberty in either male or female rats.

There are a number of instances of pregnancy following thymectomy in the female. None of them, so far as could be discovered, were in animals which were examined for fragments of thymus later. They are reported by Fischl (1907) who produced 2 broods of chicks from thymectomized hens, one of which was mated with an operated rooster; by Paton and Goodall (1904) in 2 guinea pigs; and Nordmann (1914) in 2 dogs. Paton (1905) reported that the first pregnancy occurred at about the same time in 8 operated and 7 control guinea pigs. In the experiments of Andersen (1931) a group of rats which had been thymectomized at 21 days were mated. Of 12 operated males, 9 were found to be fertile, while 8 of 11 control males were fertile. Of 16 operated females 12 were fertile, while 14 of the 15 controls were fertile. The litters were grown to adult life.

From an analysis of this work on thymectomy we may conclude that thymectomy does not prevent, hasten or delay the arrival of sexual maturity, and it does not prevent the occurrence of normal litters. In other words, aside from the immediate effects of the operative injury, it has not been shown that deprivation of thymus, even in early life, has any effect upon the development and function of the sexual apparatus.

The effect of the administration of thymus substance on the gonads. A. The feeding of whole thymus. Most of the work on thymus feeding has been on tadpoles and amblystoma. It has been fully reviewed by

Romeis (1925) and Gudernatsch (1930) and only a brief summary will be given here. Beginning in 1912 Gudernatsch reported a series of experiments in which he fed tadpoles with various glands of internal secretion. The two glands which produced striking results were the thyroid, which hastened metamorphosis, and the thymus, whch had irregular results but on the whole produced large tadpoles which metamorphosed late or not at all. Romeis (1914) confirmed this. Uhlenhuth (1918) pointed out that if known quantities of thymus were fed to one group of amblystoma larvae and the same quantity of worms were fed to controls, the thymus-fed animals metamorphosed first, but if the quantities were unlimited the thymis-fed animals grew well and metamorphosed somewhat later. Romeis in 1925 confirmed again the finding that growth and development were delayed in tadpoles by an exclusive diet of thymus if they were very young, but not if they were well started on metamorphosis. Moreover, if they were given supplementary muscle or plant food in addition to thymus even the very young tadpoles developed normally. He found no evidence for a specific hormone, but considered that the thymus as a food was deficient in some substance necessary for metamorphosis. These findings were confirmed by Abderhalden (1926). The animals used by each of these workers numbered into the hundreds and were well controlled by the feeding of other organs.

The mammalian experiments are fewer in number. The first that appeared were those of Hewer (1916) on rats. She fed thymus tablets to a group of 7 males and 4 female albino rats; fresh lamb's thymus to 5 males and 3 females; and kept 6 males and 6 females as controls. The experiment was begun at the age of 3½ weeks and no rats weighing less than 25 grams at the time were used. They were fed their daily ration of thymus first, and then "ordinary food," type not specified. The growth rates were alike in the 3 groups. In male animals fed *small* amounts of thymus daily (4 on tablets and 3 on the fresh gland) there was delay in the appearance of spermatogenesis to the extent of one week. That is, assuming 10 weeks as the normal age at which mature spermatozoa first appear in the epididymis, the thymus-fed rats killed at 10 weeks showed spermatogenesis in the testis but only occasional mature sperm in the epididymis, a condition which the author considered normal at 9 weeks. In a group of 7 male rats fed *large* quantities of thymus with the addition of a little milk, only a few spermatozoa were present at 10 weeks, and in animals killed at 19½, 38½ and 41 weeks spermatogenesis was absent. The thymus in all these cases

appeared normal. In the females the age at the first litter was 13 to 14 weeks in the control and tablet-fed groups, but 14 weeks in the fresh-thymus-fed group. The length of gestation of the various groups was given as: controls (10 rats) 23 to 38 days, tablet fed (8 rats) 24 to 34 days, fresh-thymus-fed (9 rats) 23 to 32 days. She concluded that there were no constant changes ascribable to the thymus feeding. These experiments are open to several criticisms. The age at puberty varies in all animals, and personal experience has shown that in littermate normal rats it may differ as much as 24 days. A series as short as the one presented with only a 10 day variation is not convincing. The work of Romeis suggests that a diet consisting exclusively of thymus is deficient in something necessary for the development of the tadpole. This may well be true for the rat, and if this substance is also absent from milk, the fact that a diet consisting of thymus and a little milk produces absences of spermatogenesis shows only that it is a deficient diet. This is a possible explanation for the absence of spermatogenesis as late as 41 weeks. In the experiments on female rats the variation in length of gestation is more extreme than the work of Evans and others has shown to be, and may have been due to inaccurate observation or possibly to inadequate diet. The work of Hoskins (1916) also on a short series of rats showed normal sex glands after thymus feeding. Gudernatsch (1918), without giving protocols, states that thymus-fed rats are fine breeders. Romeis (1926) checked his findings in the tadpole on the rat and reported marked delay in the development of the sex glands on an exclusive thymus diet. The testes in these rats were one-eighth the normal weight and showed no spermatogenesis. If the thymus diet was supplemented with vegetable food rich in vitamines, the rats and their sex glands were normal. These experiments of Romeis seem final and convincing.

The experiments of Soli (1910) and of Riddle (1924) come under the heading of calcium metabolism rather than reproduction in the strict sense. Soli thymectomized 4 hens and had as controls 2 partially thymectomized birds and one normal one. About 2 weeks after the operation the thymectomized hens began laying soft-shelled eggs and continued to do so for a week, after which a few thin-shelled and then normal eggs were laid. There was no note on the diet. Riddle fed thymus to some of his pigeons which had abnormalities of the eggs associated with the lack of albumen and shell; they recovered promptly. The recent work of Ackert and Morris (1929) and especially of Morgan and Grierson (1930) on thymectomized chickens gave negative results.

The care given to feeding in the latter experiment may account for the difference.

It may be concluded that the weight of evidence is against any specific effect of the thymus as a food, at least in amphibia and mammals, providing that the animals are well cared for and given an adequate supplementary diet.

B. The effect of thymus extract on the sexual apparatus. Experiments with thymus extract have made very few contributions to the solution of the problem of thymus-sex relationships. Since we are in the dark as to what a potent extract might be expected to do, we have no criteria as to the method of preparation. It seems futile, therefore, to analyze in detail the experiments of this type. Very few workers mention the sex glands of their injected animals. In many of the experiments the animals die, soon after the injections, of "thymus death" which strangely resembles death from protein shock. Downs and Eddy (1920) using a saline suspension of pulverized desiccated thymus found no changes in the sex glands, but even a negative experiment is unconvincing because a possible "specific substance" may have been destroyed by the desiccation.

A group of recent French workers (Camus and Gournay (1924), Lereboullet and Gournay (1927), Mandelstamm (1927)) have reported a few cases in dogs and men of delayed development of the testes, especially cryptorchidism, cured by the injection of thymus extract. The cases are few and not well controlled.

The use of thymus extract to decrease the fatigue of the uterine muscle in labor was suggested by Temesváry in 1925. His experimental work (1926) on the effect of various commercial extracts of thymus on the uterine muscle of 8 guinea pigs and 12 pregnant women showed that the extract had no effect on non-pregnant or pregnant uteri until after labor had begun, but strengthened and prolonged the contractions in the first and second stages of labor. This effect was much increased by the addition of pituitary extract, and the action of the latter was modified so as to prolong the contractions and make them more rhythmical. Knaus (1926) pointed out that very large doses of thymus must be used and that in "physiological" doses there was no effect. Kohler and Porges (1927) substituted extracts of other organs for the thymus in connection with pituitrin and found that the ovarian, mammary and placental extracts worked as well, so that the effect does not seem to be one which is produced by thymus extract exclusively. The combination of thymus and pituitary extract is called thymophysin. It has been used suc-

cessfully to increase the uterine contractions during the first and second stages of labor by clinicians in Germany and America, and many hundreds of cases have been reported. The best review from the clinical point of view is that by Jarcho (1930).

The findings of Deuticke and Graff (1929) are analogous. They showed that if thymus extract was injected with ovarian hormone in the Allen test, only one-third as much of the ovarian hormone was needed. This is not a specific action of the thymus, however, as muscle extract has the same effect.

Effect of adult gonads or blood serum on the thymus. In 1911 Fiori and Franchetti injected the blood serum of old rats and rabbits into immature animals of the same species and obtained various symptoms, among them tremors, skeletal changes and death. They also found premature involution of the thymus with decrease in the small thymic cells in their animals. The experiments were repeated in 1914 with a few more animals and the same results. They controlled the experiment by injecting the blood of young animals into other young animals; there was no change in the thymus. The experiments are suggestive but difficult to evaluate because of the small number of controls. Trentini (1926) injected blood from rabbits castrated at about 13 months and used a year later, when the thymus was involuted, into 35 day old rabbits and found an increase in the size of the gland. Only 4 rabbits and 4 controls were used. He interpreted these results as due to the activity of the hypophysis which is hypertrophied after castration. Da Re (1929) transplanted adult ovaries into young spayed rabbits. The transplants grew and the thymus showed premature age involution. Kinugasa (1930) transplanted a series of adult rat gonads into immature rats of the same sex, using 4 males and 6 females with 3 male and 6 female controls. He then killed the animals at 53 and 54 days of age, 8 to 18 days after the operation, and calculated the relative volumes of the cortex and medulla. He found that the weight of the thymus was about the same in the experimental and control animals, with a slightly lower relative volume of cortex in the former.

In all of these experiments the numbers of animals used are so small as to leave the final results in doubt. No contradictory experiments were found, and it is conceivable that transplants of adult ovaries and testes and injections of adult blood serum in young animals do hasten the involution of the thymus. It would have to be proven that this was neither the effect of the operation nor of the injections on the general state of nutrition, and experiments which are thus adequately controlled have not been done.

Criteria of experiments on the thymus and the gonads. In view of the fact that the great majority of the experiments covered by this review have been carried out in such a way as to make the conclusions worthless, it seems expedient to draw attention to some of the pitfalls by the way.

1. Experiments in which the answer is in terms of the weight of the thymus under various conditions, should include enough animals so that the large normal variation in thymus weight is accounted for, and the results should be treated statistically with calculation of the probable error. The fact that under-nourishment, acute infection, age, pregnancy and possibly the injection of foreign protein all decrease the weight of the thymus, must be considered. Moreover, the relative weight in proportion to body weight is significant.

2. Experiments in which the answer is in terms of the weight, microscopic appearance or function of the gonads, should also include a large series of animals. The age of puberty, for example, is variable and varies still more with the change of season. Many aspects of gonadal function are closely related to general nutrition and to the presence of certain vitamines (Evans and Bishop, 1923; Sure, 1927), so that the diet should be carefully considered and fully noted.

3. In experiments involving operative procedures it should be remembered that both the gonads and the thymus are affected by infection and by the general condition of the animal.

4. In experiments involving injection of material the effect of introducing large quantities of foreign protein should be controlled.

5. In experiments involving introduction of material through the digestive tract an adequate supplementary diet is essential.

CONCLUSIONS

1. The thymus gland increases in size until about the time of puberty, when it undergoes involution. The microscopic picture is typical in the various age periods. During involution there is decrease in cortex, and to a lesser extent of the medulla, with replacement by connective tissue and fat.

2. The normal age involution is probably hastened by mating, pregnancy and lactation. There may be regeneration after pregnancy.

3. Castration or spaying of young animals delays, but does not prevent age involution.

4. Thymectomy has no effect on the morphology or physiology of reproduction, except through trauma or infection from the operation, so far as has been demonstrated.

5. The administration of thymus gland has no specific effect on reproduction other than that of a good food.

6. The administration of thymus extract has no specific effect on reproduction, so far as has been demonstrated.

7. The transplantation of adult gonads into young animals probably hastens age involution. The injection of adult blood serum into young animals may hasten age involution.

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PHOTODYNAMIC ACTION

HAROLD F. BLUM

From the Division of Physiology, University of California Medical School, Berkeley, California

Photosensitization to light by substances not normally present in biological systems of their environment is commonly designated as *photodynamic action.* This use of the term originated with Tappeiner (112) who, together with his students, contributed a great deal to the early investigation of the phenomenon, and although a broader interpretation of the term has been suggested,[1] it seems wise to use it in the restricted sense in which it has been applied to biological reactions. Under the term are included many examples of experimental sensitizations to visible light by fluorescent dyestuffs and pigments, e.g., the hemolysis of red blood cells by light and eosine or chlorophyl; sensitization by the ingestion of sensitizing substances as in poisoning of domestic animals by buckwheat or St. Johnswort; and at least some of the numerous light sensitizations which are observed from time to time in the clinic. The term *photosensitization* has often been applied to these phenomena, quite appropriately, since they undoubtedly depend upon photosensitizations in a stricty chemical sense; there are, however, objections to the unqualified use of the term. As will be shown below, the photodynamic phenomena are all dependent upon chemical reactions of fundamentally the same character, whereas other photosensitized reactions occur normally in living organisms, as for example, photosynthesis, which involve quite different secondary chemical reactions although resulting from the same primary photo process. In our present state of knowledge of light mechanisms in biology it seems therefore convenient to use the term *photodynamic action* to designate the particular group of phenomena outlined above which form a more or less distinct entity.

The phenomenon was discovered in the winter of 1897–98 by O.

[1] Sellards (103) has suggested that the term be applied inclusively to the effects of light on biological systems. This has, however, not been widely adopted.

Raab[2] (95), working under the direction of H. Tappeiner. He was studying the pharmacological effects of acridine, and in attempting to determine the lethal dose of this substance for paramecia, found that if the acridine solutions containing the paramecia were exposed to light, the organisms died much more rapidly than if they were maintained in darkness. Light alone did not kill the organisms, however, showing that the lethal effect was due to the combined action of acridine and light. Raab's finding immediately aroused considerable interest, and the phenomenon was intensively studied in the years immediately following, particularly in Tappeiner's laboratory in Munich. The interest has continued to the present time and has spread to investigators in numerous fields, with the result that more or less sporadic investigations have been carried on, and a diverse group of phenomena have been described as photosensitized by various substances, principally fluorescent dyestuffs, but including some naturally occurring pigments such as chlorophyl and hematoporphyrin. Certain of the individual phenomena will be discussed in a later section of this paper, but it will be our plan to consider first what is known of the fundamental process underlying the entire group.

THE NATURE OF THE PHOTOCHEMICAL PROCESS INVOLVED. In photosensitized reactions the sensitizing substance acts as the light absorber, which, while remaining itself unchanged, brings about reactions of other components of the system which themselves are not affected by light. In accordance with the Grotthus-Draper absorption law, which states that only light which is absorbed can produce chemical change, the active radiation is limited to those wave lengths which are absorbed by the sensitizer. Modern photochemical theory accepts the Stark-Einstein equivalence law for the explanation of the primary photochemical process. This states that molecules absorb radiant energy in discrete quanta, the magnitude of the quanta depending upon the frequency of the absorbed light. The quantum of absorbed energy E is equal to $h\nu$, where h is Planck's constant and ν the frequency of the absorbed radiation. It would be outside our province to discuss here the possible changes in the absorbing molecule other than to state that in the reactions with which we are dealing the primary act is to raise a valence

[2] Marcacci (79) described the poisoning of germinating seeds, green plants, fermentation processes, and developing frog's eggs, by quinine and cinchonamine in the presence of light. He did not, however, recognize the significance of his findings.

electron to a higher quantum orbit resulting in an activated molecule.[3] The primary reaction may be represented thus.

$$S + h\nu = S'$$

where S is the normal molecule of the sensitizer and S' the activated molecule.

The activated molecule which is extremely short lived may take part in chemical reaction upon collision with some other molecule in the system, or may lose its energy by emitting light as fluorescence or as kinetic energy through collision with other molecules. It may possibly transfer its activation to another molecule which can then react chemically. The number of the activated molecules actually taking part in chemical reactions subsequent to the primary reaction and the kind of reaction which takes place is determined by the concentration and kind of molecules present in the system.

The active radiation. From the above discussion it is evident that if the photodynamic phenomena are dependent on photosensitized chemical processes, the active radiation must fall within the absorption range of the particular sensitizer which is used. That this is actually the case has been demonstrated in numerous instances, which while not covering all the known photodynamic processes, represent sufficiently typical cases. Raab (95) made a considerable number of critical experiments before the first publication of his work, among them the approximate determination of the radiation active in the photosensitization of paramecia by acridine. By filtering out various portions of the spectrum with colored solutions, he arrived at the conclusion that the active radiation was that absorbed by the acridine. A filter of acridine itself completely prevented the destruction of the organisms. Hertel (47) found that light of 518 mμ destroyed bacteria, and paramecia sensitized with eosine or erythrosine, whereas 448 mμ did not. The former radiation lies within the absorption spectrum of these dyes, while the latter does not. Metzner (84) found an approximate correspondence of the active wave lengths to the absorption spectrum for a number of dyes. Lippay (73) found that sensitized skeletal muscle was stimulated to contraction only by those radiations absorbed by the sensitizer. Supniewsky (107) found rabbit's intestine was sensitized by hematoporphyrin to only wave lengths which this substance absorbs. Karschulin (64) shows a general correlation between the

[3] For a discussion of this subject the reader is referred to Risse (97) or Kistiakowsky (66).

photodynamic activity and the absorption spectrum, together with certain unexplained variations. Dreyer (23) found that sensitization of infusoria by erythrosin was apparently uniform throughout the visible spectrum. However, the actual wave lengths and intensity of the light passing his filters cannot be estimated from his data, and since the dye has a wide absorption in the visible, it seems probable that sufficient light was absorbed by the sensitizer in all cases to account for the reactions.

Apparent minor deviations in the active radiation from that of the absorption spectrum of the dye in aqueous solution are to be expected. Metzner (84) finds that the absorption spectrum of certain dyes is considerably shifted toward the red by combination of the dye with the protoplasm of the cell. In such a case it may be necessary to consider the absorption spectrum of the dye protoplasm combination and not simply that of the dye in aqueous solution. In certain cases the absorption spectrum is considerably shifted by hydrogen ion concentration, which may be different within the cell and in the surrounding medium. This might account for apparent deviation in some instances.

Gros (33) studied the effect of concentration on the rate of bleaching of fluorescein dyes in light. He found that at a given concentration of dye the rate of bleaching was at a maximum, increase or decrease in concentration above this value being accompanied by a decrease in the rate. Likewise, Jodlbauer and Tappeiner obtained similar results for the sensitization of Eder's solution (57), the oxidation of iodide ion to iodine (60), the destruction of invertase (60), and Fr. Tappeiner (111) for the hemolysis of erythrocytes; these results are summarized by Tappeiner (110). Since light absorption is proportional, within limits, to the concentration of absorbing substance,[4] it should be expected that the reaction would proceed faster with increased concentration of the sensitizer up to the point of complete absorption, since greater numbers of molecules would be excited. However, the absorption of light in successive layers of solution should follow a logarithmic function,[5] so that the greatest absorption must be in the layer of solution nearest the source of radiation, and in concentrated solutions the greater part of the absorption may take place in this surface layer. If the rate of a resulting chemical reaction is limited by the concentration of some

[4] Beer's Law.

[5] Lambert's Law.

These laws could not be expected to hold exactly in systems of the type described but may be assumed to do so for this illustration.

reacting component in the surface layer other than the absorbing substance, only a part of the light energy absorbed in the first layer of solution may contribute to the bringing about of chemical changes. In the less concentrated solutions, the absorption in the surface layers being less, a larger part of the light energy might be used in bringing about chemical changes in the deeper layers of solution. The limiting factor in Gros' reactions was undoubtedly, as he concluded, the concentration of dissolved oxygen which was limited by the rate of diffusion of oxygen to the surface layers. In systems such as suspensions of red blood cells, the limiting factor is probably the concentration of cells in the first layers, if these are distributed uniformly throughout the solution. Red cells themselves carry a considerable amount of loosely bound oxygen, but in other biological systems the diffusion of oxygen may be the limiting factor; since, as will be shown, the photodynamic reactions involve molecular oxygen.

The absorption spectrum of sensitizing dyes is not usually uniform; and we might expect that if these reactions were studied with monochromatic light, the wave lengths producing the greatest rate of chemical change would differ from those showing maximum absorption by the dye when relatively high concentrations of the dye are present, since the penetration of light would be greater for wave lengths other than those corresponding to the maximum absorption. This may well be the cause of the apparently anomalous results obtained by Dognon (20) who studied the effect of certain emission bands of the mercury arc, finding that the maximum photodynamic effects (killing of paramecia) did not correspond to the maximum absorption of magdala red. Dognon (21) points out other variations in the relationship of concentration to photodynamic activity; he finds, however, a linear relationship between photodynamic action and intensity of radiation at a given concentration.

It is, of course, necessary to consider the direct destructive action of other wave lengths than those absorbed by the sensitizer in experiments on living organisms. Radiation in the ultra violet, particularly of wave lengths less than 290 mμ, is active in bringing about destructive effects in biological systems which are similar to photodynamic effects. For instance, hemolysis of red blood cells is brought about by such radiations in the absence of a sensitizer or by the appropriate visible wave lengths when a sensitizer is present. The processes appear to be, however, fundamentally different as will be shown below. Ordinarily the destructive ultra-violet radiations are screened out by glass when

experiments are performed on sensitized systems. It is probable that the sensitizers themselves act in most cases as filters for the ultra-violet, since they usually show general absorption in this region. This appears true from the experiments of Pessoa and Pereira (92) who found that paramecia in eosine solution were not killed when exposed to light from a quartz mercury lamp. The writer has likewise found that red blood cells in agar plates containing eosine show less hemolysis after a given exposure to quartz mercury arc irradiation than similar plates not containing the dye. On the other hand, Gassul (31) claims to have sensitized mammals to ultra-violet radiation by means of eosine.

Sensitization to wave lengths in the ultra-violet by sensitizers absorbing in this region is, of course, quite possible. Hausmann and Sonne (45) and Lassen (69) claim that sensitization of red blood cells to light in the ultra-violet is brought about by hematoporphyrin, although Fabre and Simonnet (25) found that only visible light was active under these conditions. Hausmann and Krumpel (41), (42), (43) have found that porphyrins and trypaflavin absorb in the ultra-violet, from which they suggest that the sensitizing action of these substances may occur in this region. Their observations indicate that these substances may possibly, but not necessarily, sensitize biological systems to ultra-violet radiation.

It is of historical interest to note that Jodlbauer (51) studied the effect of x-ray and radium on paramecia and enzymes, treated with eosine. Viale (115) and Kammerer and Weisbecker (63) conducted similar experiments. The results were negative in both cases.

The irreversibility of the process. Efimoff (24) has demonstrated that the killing of paramecia by light and eosine obeys Talbot's law. Two samples of paramecia sensitized with eosine were exposed, one to continuous light, and the other to intermittent light consisting of five minute intervals of light and darkness. The organisms were killed by the same total period of irradiation, i.e., exposure to the same quantity of energy in both cases. Thus the reaction is irreversible, no recovery taking place during the intervals of darkness.

The oxidative nature of the process. The photodynamic phenomena may be ultimately described as oxidations by molecular oxygen. Whatever the actual reactions following the primary excitation of the sensitizer, considerable evidence has accumulated to indicate that the presence of molecular oxygen is required for these reactions. Ledoux-Lebard (70) found that the killing and cytolysis of paramecia by eosine and sunlight occurred more rapidly if the organisms were exposed in

shallow dishes than when exposed in deep tubes. He concluded that this was due to the fact that oxygen was required for the reactions involved, more oxygen being available in the shallow vessel than in the deep one. Following this suggestion, Jodlbauer and Tappeiner (58) found that the photodynamic destruction of bacteria by rose bengal, methylene blue, and phenosafranine was inhibited by the lack of oxygen. Hasselbalch (36) found that hemolysis by light with eosine, methylene blue, and other dyes was inhibited in a vacuum. Metzner (81) found that induced phototropisms and killing of protozoa, spirilla, and diatoms brought about by photosensitizers is dependent upon the presence of oxygen. Schmidt and Norman (101) found that hemolysis by eosine and light does not take place in an atmosphere of hydrogen. Lippay (74) finds that oxygen is necessary for the photodynamic stimulation of striated muscle, and Clifton (16) that the destruction of bacteriophage by light and methylene blue does not take place in a vacuum or in nitrogen. Hausmann and Löhner (44) found that some, but not all, of the symptoms of photodynamic action on white mice were inhibited by reduction of the surrounding atmospheric pressure. Blum and McBride (8) found that photodynamic hemolysis by eosine was completely inhibited in pure CO, but that in mixtures of 80 per cent CO with 20 per cent O_2 the hemolysis took place as in air.

It is interesting at this point to note that the requirement of oxygen for the photodynamic phenomena separates them distinctly from the destructive effects of ultra-violet light on biological systems. Bie (5), after careful consideration of the existing evidence, concluded that the destructive effects of ultra-violet as a whole do not require molecular oxygen. Forbes and Daland (27) found that the killing of paramecia by ultra-violet light did not necessarily involve oxygen. Jodlbauer and Tappeiner (58 and 62) found that the destruction of enzymes by ultra-violet occurs in the absence of oxygen, whereas the photodynamic destruction of these substances occurs only in the presence of oxygen. Hasselbalch (36) showed in a careful study that hemolysis of red blood cells by light from the quartz mercury arc takes place in the absence of oxygen, in contrast to his finding that oxygen was required for photodynamic hemolysis under practically the same experimental conditions.

The oxidative nature of the process is also indicated by inhibition by reducing agents. Sacharoff and Sachs (98) found that the presence of sodium sulfite prevented photodynamic hemolysis. Noack (88) showed that sodium sulfite may inhibit other photodynamic effects. Schmidt and Norman (101) found that those readily oxidizable substances which

react with the Folin Denis reagent also protect red blood cells from hemolysis by eosine and sunlight; among these substances are tyrosine and tryptophane.

Since serum protects red blood cells from photodynamic hemolysis and Busck (9) has shown that this may be attributed to the protein constituents of the serum, Schmidt and Norman (101) suggested that the protective effect is due to the proteins containing tyrosine and tryptophane. Rask and Howell (96), however, found that euglobulin, pseudoglobulin, egg albumin, and peptone, all of which contain tyrosine, are not active in inhibiting photodynamic action. By careful separation of the constituents of serum, these authors found that the serum albumin is the principal substance responsible for the protective effect; their test of photodynamic action was the damaging effect of light on the terrapin heart perfused with hematoporphyrin. Viale (113, 114, 116) found that a number of readily oxidizable substances inhibited photodynamic hemolysis but obtained some irregular results, particularly with serum and with casein. Awoki (3) found that injection of serum protected white mice against the fatal effects due to sensitization by hematoporphyrin, whereas inorganic reducing agents did not. Lippay (74) found that horse serum inhibits the stimulation of frog's striated muscle by sensitizers and light. He observed that in such solutions the muscles were much less colored by the sensitizer, and suggests that inhibition in this case is due to the fact that serum hinders the penetration of the sensitizer into the muscle. It seems likely that combination of the dye with the protein may be at least a part of the mechanism of the protective action of proteins and of serum. It is, of course, probable that the observable photodynamic effects may be inhibited in numerous ways not necessarily connected with the photochemical processes involved, since alterations in any part of the complex biological system may have its effect on the total result; however, the fact that numerous simple, easily oxidizable substances exert a protective effect is strong evidence that the photodynamic phenomena involve oxidations. Dognon (22) recognizes three types of desensitization or inhibition of photodynamic effects, *a*, by proteins due to combination with the sensitizer, *b*, by reducing substances, and *c*, by the desensitizing action of dyes such as phenosafranine. The latter is comparable to desensitization of the photographic plate, the mechanism of which is not definitely understood, but has been explained as an oxidation-reduction process (see 11).

A considerable number of oxidations involving atmospheric oxygen

are known to be photosensitized by substances of the type with which we are dealing. Among the first of these to be studied was the oxidation of iodide ion to iodine, which was investigated by Straub (105 and 106) and Jodlbauer and Tappeiner (58). This reaction goes on at a very slow rate in the absence of light, except in acid solutions. The rate of oxidation is increased by radiation in the ultra-violet, but not by visible light except in the presence of a sensitizer. Straub (105) pointed out the analogy between the oxidation of iodide and photodynamic phenomena, and suggested that the latter were due to the oxidation of cell constituents brought about by light and a sensitizer. This simple hypothesis was set aside by the speculations of Jodlbauer and Tappeiner (58 and 110), but was later revived by Noack (88). Blum (6) found a still further analogy between the processes by showing that the oxidation of iodide and the hemolysis of red blood corpuscles could both be brought about by previously irradiated sensitizers and that the two processes showed certain similarities. Jodlbauer and Tappeiner (58) added certain other inorganic and organic oxidations as reactions photosensitized by fluorescent dyes; and Gaffron (28) demonstrated the uptake of oxygen by tyrosine, phenol, and uric acid when sensitized to light. Carter (12) studied the oxygen consumption in the photosensitized oxidation of a considerable number of organic substances using dyes or hematoporphyrin as sensitizers. He found that only ring compounds could be oxidized in this fashion, and among these only those compounds which contained a hydroxy or amino group. Such compounds are known to be readily oxidized by various oxidizing agents and may often show an appreciable oxidation in air. It seems that we may possibly look to compounds of this type as the point of attack in photodynamic action.

The oxidations through photosensitizations by heavy metals, which have been demonstrated by Neuberg, are of questionable importance in relation to our studies; Neuberg and Galambos (87) found that very few of the same oxidations were brought about by the fluorescent dyestuffs which are active in producing photodynamic effects. Noack (88) found that he could not produce certain photodynamic effects, e.g., the killing of paramecia, by means of metallic sensitizers, and Viale (113) made similar observations with regard to hemolysis of red blood cells.

The oxidative destruction of various constituents of living systems has been demonstrated. Jodlbauer and Tappeiner (58 and 61) found that certain enzymes and toxins were destroyed by photosensitized

processes, but only in the presence of oxygen. Hasselbalch (36) showed that hemoglobin is oxidized to methemoglobin when sensitized with fluorescein dyes, but only in the presence of oxygen. Noack (88) showed that certain plant pigments can be oxidized by various photodynamic substances and light, the change of color in the pigments themselves being used as an indicator of the oxidations. Baumberger, Bigotti, and Bardwell (4) found that methylene blue and light inhibited the coagulation of a mixture of fibrinogen, calcium, and prothrombin, but only in the presence of oxygen; CO_2 was given off in the process and O_2 absorbed.

A mechanism for these oxidations was suggested by Straub (106). He suggested that the photosensitizer combines with molecular oxygen to form a peroxide, due to the action of light. This peroxide then reacts with the oxidizable substance bringing about its oxidation, the peroxide itself being reduced to the original sensitizer in the process. Straub based his argument on the fact that oxygen was required for this reaction, and that iodide ion equivalent to many times the quantity of dye present could be oxidized in this way. This suggested the cycle described above as he assumed that the sensitizer peroxide was formed over and over again from the small quantity of sensitizer present. Gaffron (28) found that in the photosensitized oxidation of serum and amino acids the oxygen uptake represented several times the quantity necessary to completely oxidize the dye. In the presence of an oxidizable substance the color of the sensitizer is retained, but in the absence of such a substance the sensitizer itself is rapidly oxidized and the color disappears. This evidence all points to some such cyclic process as suggested by Straub.

Gaffron (28) could not demonstrate the actual formation of dye or chlorophyl peroxide. He suggests that the sensitizer acts as an oxygen transporter to the acceptor, i.e., the oxidizable substance. As to the actual formation of intermediate peroxide, Gaffron (30) has shown quite definitely that peroxides are formed when chlorophyl is irradiated in solution in isoamyl-amine, but concludes that this is not a peroxide of chlorophyl. Using Warburg's method, he measured the oxygen absorbed during irradiation; at the end of the period of irradiation MnO_2 was added to the solution and the O_2 released was measured. There was a very close correspondence of these two quantities, indicating that the O_2 absorbed was bound as peroxide oxygen. Likewise, he determined this peroxide by adding KI in the dark, and titrating the iodine formed; this method also gave a very close approximation to the O_2 bound during

irradiation. He found that the quantity of peroxide formed might be several times the amount equivalent to the quantity of chlorophyl present, and concludes, therefore, that the peroxide which is formed is not a peroxide of the chlorophyl, but probably H_2O_2 or a peroxide of the amine. This evidence does not preclude the formation of a sensitizer peroxide as an intermediate step in the oxygen transport. Blum (6) found that after irradiation of solutions of the fluorescein dyes, the addition of KI in the dark resulted in the oxidation of iodide ion to iodine. This indicates the formation of a peroxide which in this case may be a peroxide of the dye, H_2O_2, or a peroxide of some of the oxidation products of the dye. In these experiments the quantity of peroxide formed, as indicated by titration of the iodine, was never equivalent to the quantity of dye present.

Gaffron (29) found that a nice confirmation of the Stark-Einstein equivalence law could be demonstrated with the absorption of O_2 by an irradiated solution of ethylchlorophylid and ethyl-thiourea in acetone. He showed that one molecule of O_2 was absorbed per quantum of light energy. This high quantum yield suggests a comparatively simple chemical process following the primary activation of the sensitizer molecule.

Neuberg and Galambos (87) suggest that the reaction concerned in photo-oxidations brought about by dyes is due to the formation of a quinone-hydroquinone system, although they also admit the possibility of peroxide formation. The greater probability of the latter mechanism is indicated by the above mentioned experiments of Gaffron.

The formation of peroxides at some point in such reactions seems clearly demonstrated. The probability that the photodynamic effects are brought about by the direct action of peroxides is indicated by the fact that effects similar to those brought about by sensitizers and light can be produced by hydrogen peroxide. Noack (88) showed that in the absence of light hydrogen peroxide produced results very similar to the effect of light and fluorescent dyestuffs. He found also that the addition of manganese salts increased the photodynamic effects of the fluorescent dyestuffs, indicating that a peroxide was concerned in these reactions, since manganese has a strong catalytic action on the oxidation of many organic substances by hydrogen peroxide. Blum (7) has shown that hydrogen peroxide may produce hemolysis when in proper concentration and also fixation of the red blood cells at higher concentrations, both of which effects are produced by irradiated fluorescein dyes; the effect is increased by the addition of small quantities of these dyes.

This latter effect will be discussed below. Carter (12) found that while the killing of *Glaucoma* by H_2O_2 could be prevented by catalase, the latter did not protect the organisms against the action of hematoporphyrin and light. Likewise, he found that fluid from tubes containing *Glaucoma* culture and hematoporphyrin which had been exposed to light gave no test for hydrogen peroxide when tested with guaiacum and peroxidase. He concludes therefrom that no H_2O_2 is formed in the photochemical reaction. This would not preclude the presence of organic peroxides which are known to be more active in bringing about oxidations than H_2O_2 and which are not destroyed by catalase. The fact that cyanide does not influence photodynamic action (8), although inhibiting catalase, indicates that H_2O_2 is not formed in such processes.

Other photochemical reactions of fluorescent dyes. The photosensitizing substances with which we are dealing bring about other types of reactions than the oxidations described above. The sensitization of photographic emulsions was the first of these processes described, but its mechanism is still quite obscure. This reaction is ultimately a reduction and does not involve molecular oxygen. The sensitization of Eder's reaction was described by Jodlbauer and Tappeiner (57). This reaction consists in the precipitation of Hg_2Cl_2 from a solution of $HgCl_2$ and ammonium oxalate. They found that a large number of fluorescent dyestuffs sensitize this reaction which is inhibited by the presence of O_2.

These reactions have apparently no bearing on the reactions which bring about photodynamic effects; which, as stated, are ultimately oxidations by molecular oxygen. They are, however, of interest, since attempts have been made to explain all these reactions on a common mechanism (36 and 110). This was due to the failure to make the distinction between the primary activation process and the subsequent chemical reactions. As previously stated, the type of reaction brought about by the activated molecule depends upon the other components of the system. It is interesting to note that chlorophyl, a natural pigment, sensitizes the photographic plate, the oxidations involved in photodynamic processes and also some part of the reactions concerned in photosynthesis; although the chemical reactions produced in the three cases are quite dissimilar.

Photodynamic substances take part in another interesting type of photochemical reaction which is not a true sensitization in that the light absorbing substance is itself altered at the end of the reaction. Levaillant (71) describes the oxidation (dehydrogenation) of various polyalco-

hols and of oxalic acid upon irradiation in the presence of fluorescein, thiazine, and oxazine dyes, provided oxygen is excluded from the reaction; these reactions are accompanied by the bleaching of the dye due to reduction to the colorless leucobase. The color returns more or less completely in the dark in the presence of oxygen. Chakvararti and Dhar (13) and Windaus and Borgeaud (120) have described similar reactions. Carter (12) found similarly that tyrosine, phenol, or guanine decolorized methylene blue in vacuum in light. In these cases the dye is acting apparently as a hydrogen acceptor, allowing the oxidation of the substrate. The reactions of ergosterol and eosine are of particular interest. Windaus and Brinken (119) found that when ergosterol was exposed to sunlight with eosine in the presence of O_2, a peroxide of ergosterol was produced. On the other hand, Windaus and Borgeaud (120) found that when the same system was irradiated in a vacuum, the ergosterin was dehydrogenated. It would seem possible from these reactions that destructive effects in the cell might be produced under proper conditions by photodynamic substances in the absence of oxygen, but this has not been demonstrated (8).

The bleaching of these dyes is of interest. As was shown above, the bleaching may take place by reduction to the leucobase, this reaction being more or less completely reversible (71). Gros (33) showed that the leucobases of fluorescein dyes are autocatalytically oxidized to the colored dyes upon irradiation in the presence of oxygen, by the sensitizing action of the dyes themselves. Thus we could not expect the above reactions to take place in the presence of oxygen. Gros has also shown that in the presence of oxygen the dye itself is oxidized to colorless compounds by the action of light. This bleaching is not reversible in the dark. That it is an oxidation is indicated by the fact that oxygen is absorbed (33) and CO_2 is evolved (13). This reversible bleaching may also be accomplished by oxidizing agents, e.g., hydrogen peroxide in the presence of traces of iron. Wood (121) describes the formation of "photocompounds," i.e., colored non-fluorescent substances, upon irradiation of some dyes; these may represent incomplete oxidation of the dye. As mentioned above, this irreversible bleaching does not take place in the presence of a readily oxidizable substance which may be oxidized instead of the dye. We see then that there are two types of bleaching of the dyes which take place, the reversible reduction to the leucobase occurring only in the absence of oxygen, and the irreversible splitting into simpler products, which is undoubtedly an oxidation. The former type of bleaching is followed by return to

the colored dye in the presence of oxygen, either in the dark or the light; the latter is not. Chakvararti and Dhar (13) state that both reduction and oxidation of eosine take place simultaneously in the light, but their evidence seems inconclusive.

The question of fluorescence. It has been stated above that one of the means by which the activated molecule may return to its normal state is by the emission of its increment of energy as light. In the case of the substances with which we are dealing, the light is emitted at a slightly longer wave length than the absorbed light, and constitutes the fluorescence typical of these substances. It would appear from this that the fluorescence actually comes from the non-reacting molecule, and represents these rather than those molecules which take part in the actual chemical reactions (see 91).

Tappeiner and Jodlbauer (112) pointed out that all those substances which produce photodynamic phenomena are fluorescent, no non-fluorescent substances being known to produce these effects. Noack (89) found that the non-fluorescent copper compound of chlorophyl did not produce photosensitization, whereas the fluorescent chlorophyl did. Likewise, Gaffron (28) found that among metallic compounds of hematoporphyrin, only those which were observed to fluoresce brought about photo-oxidation.

It was at first conceived that the photodynamic effects might be due to the destructive effects of the fluoresced light. This idea was tested by Raab (95) who showed that paramecia were not damaged when exposed to the fluoresced radiation from acridine solutions with which they were not in contact. Sacharoff and Sachs (98) repeated the same type of experiment with red blood cells and various fluorescent substances. There seems to be nothing about fluoresced light to afford it such destructive properties as it is simply light of a somewhat longer wave length than that absorbed by the fluorescent substance. Weigert has recently shown that a small portion of this light may be polarized (see 94, p. 195) but we have no evidence that polarized light may exert such destructive effects. With these facts in mind, it is interesting to find that the destructive effects of "fluoresced" radiation is mentioned from time to time in the literature.

Tappeiner could not demonstrate a correlation between the degree of fluorescence and photodynamic action. He pointed out (108) that visible fluorescence is diminished by the introduction of substituted halogen atoms into the fluorescein molecule, a rapid decrease occurring with increase in number and atomic weight of the halogens, and that

this diminished fluorescence is accompanied by an increase in photodynamic action. It will be shown later (p. 39) that this increase of photodynamic activity may not be due to an actual increase in the ability to produce photochemical change, but at least no decrease in the latter can be demonstrated to accompany the marked decrease in fluorescence. Tappeiner, nevertheless, stressed a fundamental relationship between fluorescence and photodynamic activity in all his writings.

J. Perrin developed a hypothesis which assumed a direct relationship between fluorescence and chemical activity. He suggested that the activated molecules undergo a chemical change in the process of reëmitting their excess energy as radiation. This would account for the fact that the fluoresced light is always of less frequency than the absorbed, on the basis that the emitted quantum is thus smaller than the absorbed because chemical energy has been added to the molecule. He explained the bleaching of dye solutions on this basis, which would require that for each quantum absorbed and fluoresced one molecule of dye would be destroyed. However, dye solutions retain their ability to fluoresce for periods of time during which light quanta are absorbed and emitted in numbers equal to many times the number of dye molecules present in the solution (see 66, p. 212). Viale (113) has suggested that the energy active in bringing about photodynamic effects is represented by the slight increment due to the difference between the energy value of the absorbed and the fluoresced light.

No satisfactory correlation between chemical structure and fluorescence has yet been given (see 94, pp. 214–219). Metzner (85) gives a tabulation of a considerable number of substances which have been tested for photodynamic action; the only common properties in the active substances appear in the possession of ring structures and the ability to fluoresce.

THE DARK REACTION ("DUNKELWIRKUNG"). In sufficient concentrations most of the photodynamic substances are capable of bringing about certain injurious effects in the dark which are at least similar to those brought about when the dyes are irradiated. For instance, eosine kills paramecia and hemolyzes red blood cells in the dark when in sufficient concentration. Likewise, in the absence of light, photodynamic substances inhibit the action of toxins and enzymes (112), (68), (52), and are lethal to vertebrate animals (54). These effects are all brought about by the same substances in much less concentration when irradiated. The concentrations concerned are not of the same order as shown by the following example from the writer's laboratory: Rose

bengal hemolyzed cells in the dark at a minimum concentration of 8×10^{-5} M, whereas under similar experimental conditions, but exposed to sunlight for one hour, hemolysis occurred in concentrations of 5×10^{-9} M.

The mechanism of the dark reaction. On first consideration it might appear probable that the reactions concerned are of the same character as those bringing about the photodynamic phenomena, i.e., oxidations by molecular oxygen. It might be assumed that oxidation of cell constituents by oxygen is brought about by the dyes in the dark, this process being accelerated by light; if this were true, these reactions should be inhibited by the withdrawal of molecular oxygen. Hasselbalch (36) found in a very small number of experiments that hemolysis by rose bengal in the dark was inhibited by evacuation of the air from the reaction chamber. Blum and McBride (8), however, found that hemolysis by non-irradiated eosine was not inhibited when precautions were taken to remove oxygen as completely as possible from the solution. Claus (15) found that in the absence of light dyes produced either decrease or increase in the activity of different kinds of diastase; if the reaction were an oxidation, it might be expected to produce the same effect in all cases.

Numerous investigators have suggested that the dark reaction is dependent upon adsorption of the dye by cell constituents (110), (56). Kudo and Jodlbauer (68) found that the action of non-irradiated dyes on tetanus toxin was reversed by dilution of the medium, and offer this as evidence in support of the above hypothesis. Jodlbauer and Haffner (56) assume, from studies on red blood cells, that the dye is adsorbed by the protein constituents of the cell and that this is the basis of the hemolysis and fixation which occur. Other hypotheses might be proposed and defended, but at the present the mechanism of the dark reaction seems obscure. That it is, however, a different mechanism from the photodynamic effect seems clear.

Effect of hydrogen ion concentration on the dark reaction. Jodlbauer and Haffner (56) studied the effect of hydrogen ion concentration on hemolysis by non-irradiated eosine and rose bengal (tetra-brom tetra-iodo fluorescein). They found that the hemolytic effect, considered as the reciprocal of the minimum dye concentration necessary to produce hemolysis, was least in the region of neutrality and increased as the reaction became either more acid or more alkaline. This increase was more pronounced on the acid side. In acid solutions of dye in high concentrations, fixation was found to take place instead of hemolysis;

the fixing action, like the hemolyic action, increased with increase in acidity. This effect is extremely important in the interpretation of experimental results, as has been pointed out by the writer (6), (7), (8).

The relationship between dark reaction and photo reaction. Jodlbauer and Haffner (55) summarized studies on a large number of dyes, and concluded that there is a correlation between the dark reaction and photodynamic action, i.e., the dyes which are most active in the dark are likewise most active photodynamically, although they make no attempt to account for this relationship. Jodlbauer (53) suggests an explanation in that adsorption of the dye by cell constituents is necessary in order that photodynamic phenomena may occur, and that the dark reaction may be taken as a criterion of such adsorption. Thus only those dyes which are adsorbed and consequently bring about the dark reaction are capable of photodynamic effects. Jodlbauer and Haffner's correlation is far from exact, being most convincing in the case of the fluorescein dyes which show a more or less parallel increase in photodynamic activity and dark reaction as we proceed through the series, both increasing with increase in the number and atomic weight of the substituted halogens. Jodlbauer attempts to account for variations by the suggestion that all dyes are not equally photochemically active and that only those which retain their photochemical activity after combination with the cell can produce photodynamic effects.

Jodlbauer and Haffner did not examine the effect of hydrogen ion concentration or dye concentration on the hemolytic and fixing activity of the irradiated dyes, but Blum (7) found another rough correlation between the action of irradiated and non-irradiated dyes, namely, that the hemolytic and fixing action of the irradiated dye is modified in the same way by hydrogen ion concentration as is that of the non-irradiated dye. On the other hand, recent experiments with fluorescein dyes in the writer's laboratory show that, although the dark reaction of all the dyes shifts in an orderly manner with changes in hydrogen ion concentration, the total reaction (photodynamic effect) does not. Thus, although there is a definite increase in dark reaction through the series, fluorescein, eosine, erythrosine, rose bengal, at any hydrogen ion concentration, the photodynamic effects show a marked variation. All the above observations must be considered as only approximate since the light source (sunlight) represents an unknown variable. Even in experiments performed simultaneously, the results are not exactly comparable because of the difference in the absorption spectrum of the dyes, since the relative intensity of the incident light at the various wave lengths is unknown.

The experiments of Blum (6) indicate that combination of the dye with cell constituents before irradiation is not necessary for these effects, since the previously irradiated dye is capable of producing them. This at once throws doubt on Jodlbauer's hypothesis, and Blum (7) suggests that the correlation of Jodlbauer and Haffner may be explained upon the assumption that the dark reaction and the light reaction are similar but additive effects. Thus, in general, those dyes which show the greater dark reaction would show the greatest total effects when irradiated, assuming the photochemical activity to be of the same magnitude and vice versa. With the two possible variables we should not expect the correlation to hold exactly true, as is the case. This hypothesis receives some support from the fact that the hemolytic and fixing action of H_2O_2 is greatly increased by the presence of small concentrations of non-irradiated dyes (7). It may be assumed that in this case the oxidation by H_2O_2 corresponds to the oxidations brought about by light and that the dark reaction of the dye is added to the oxidative effects in the same way in both instances. It is possible, however, that the dye may act as an activator of the H_2O_2 in the same manner as peroxidase, i.e., by combining with it to form an organic peroxide.

THE POINT OF ATTACK IN THE CELL. Thus far the discussion has been chiefly limited to the consideration of the effects of changes in cell environment upon the cell, and as we have seen, a number of extremely interesting and quite definite generalizations may be drawn from this evidence as to the type of processes involved in the photodynamic phenomena. However, as in all biological investigation, the interpretation of these processes in terms of the cell itself is a difficult and dangerous one. For instance, the evidence that the photodynamic phenomena involve oxidative processes seems established beyond question. The point at which these oxidations are effective and the resultant changes in the cell are different and much more involved problems.

The question was raised by Osthelder and Erhardt (communicated by Tappeiner, 109) as to whether the photodynamic phenomena take place at the surface of the cell ("Aussenwirkung") or necessarily within the cell ("Innenwirkung"). They attempted to determine this by treating paramecia with dyes, then separating the cells from the dye solution and placing them in a fresh solution without dye. Their results were irregular, varying with different dyes; cells stained with methylene blue, for instance, showing photodynamic reactions after removal from the dye solutions, whereas cells treated with eosine did not. Fr. Tappeiner (111) obtained similar results with blood cells.

Dognon (19) found that most photodynamic dyes did not penetrate the cell to an appreciable extent. Supniewski (107) reports that rabbit intestine takes up hematoporphyrin and is sensitive to light after removal from contact with the solution. Such varying results indicate that the penetration of the dye into the cell is not a limiting factor in photodynamic effects.

Metzner (82) arrived at the conclusion that the dye must combine with protoplasmic constituents in order to produce photodynamic effects. As evidence for this he showed (84) that the absorption spectrum of the dye in aqueous solution does not correspond with the spectrum active in bringing about induced phototropic responses in *Paramecium caudatum*, but that in the case of all the dyes he examined, the region of the wave lengths which produces the phototropic effects extends considerably farther toward the red end of the spectrum than do the absorption spectra of the aqueous solution of the dye. He found likewise that in mixtures of the dyes with egg albumin, gelatine, casein, lecithin, or with dead paramecia, the absorption spectrum is shifted toward the red. However, according to his measurements, the photodynamically active spectrum extends much farther toward the red than any of the absorption spectra measured, even that of the stained protozoa itself. This suggests that the measurement of the photodynamically active spectrum is subject to some unknown systematic error, and therefore the conclusion of Metzner that the dye must combine with cell constituents in order to be photodynamically active should be accepted with some hesitation when based on this evidence alone.

There would seem to be very little reason that the combined dye should be more active photochemically than the uncombined dye. The primary process of activation would be expected to be the same, and the uncombined molecule being the smaller might be expected to be even more reactive than the combined, because more mobile. We might predict that the reactions brought about by the combined dye would be the same as those brought about by the uncombined, the matter of combination being of only secondary importance. We should expect, of course, that the photochemical reaction would actually be more effective if it took place within the cell than if it took place at the surface because of the better chance for the activated molecules or intermediate products to come in contact with the molecules of substrate, and in red blood cells at least, because of the abundance of loosely bound oxygen in the cells. We should expect that in organisms containing pigment the photodynamic response would be slower, as has been shown by

Gicklhorn (32) and Metzner (81), because of the shielding effect of these substances, since the failure of light to reach the dye within the cell or the surface opposite the source of illumination because of absorption by colored substances in the cell would be expected to decrease the total photodynamic effect. This would be true, however, whether the dye were combined with cell substance or not, and cannot be accepted as proof of the former condition.

Jodlbauer (53) accepts Metzner's evidence in his attempt to explain the correlation between the dark reaction and photodynamic phenomena (55) by the suggestion that the dark reaction may be taken as a criterion that the dye combines with cell constituents, this combination being necessary for photodynamic action. As the writer has stated above, this correlation is a very rough one and may be readily explained on other grounds. We find that among the vital dyes which may be observed to enter the cell, methylene blue, which shows little dark reaction, is an extremely active sensitizer, whereas rhodamine B shows a comparable dark reaction but is rather weakly photodynamic. On the other hand, the fluorescein dyes penetrate very little and are easily removed by washing, but include the most active dyes photodynamically.

Certain other theories assume, like the above, that the dye and cells must be irradiated together, though not necessarily presupposing a combination of the dye with cell substance. This is true of the theory of Tappeiner which has enjoyed somewhat general acceptance. This theory, as outlined by Tappeiner in his review article of 1909 (110), assumes that the presence of the photodynamic substance merely accelerates the action of visible light. The split products of this reaction are removed through oxidation by molecular oxygen, but ordinarily these products accumulate and inhibit the reaction. The combined action of the photodynamic substance accelerates their oxidative removal and consequently the total reaction. He based this hypothesis upon the observation that visible light produced destructive reactions in certain cases similar to ultra-violet light, and so assumed the constant but suppressed action of visible radiation was allowed to proceed only when the photochemical oxidations removed the retarding substances.

Schanz (100) has put forward the theory that the photodynamic phenomena are due to changes in cell constituents due to the absorption of electrons emitted by the dye when irradiated. He arrives at his conclusions from studies on the photo-electric effect in albumin sensitized by fluorescein dyes. Clark (14), considering Schanz' findings, suggests that the sensitizer shifts the photo-electric threshold of the cell constit-

uents from the ultra-violet to the visible region corresponding to the dye absorption. It is interesting that Jodlbauer and Tappeiner (quoted from Tappeiner, 110, p. 723) attempted to determine whether the photo-electric effect was responsible for photodynamic action. They placed a hanging drop of paramecium culture as near as possible to the surface of a fluorescent solution and exposed it to sunlight for several days; the paramecia remained unharmed.

All of the above theories assume that the dye and cell must be in close proximity at the time of irradiation. The finding that previously irradiated dyes may produce phenomena altogether similar to the photodynamic phenomena is in opposition to this concept. Ledoux-Lebard (70) found that paramecia were killed and cytolyzed by previously irradiated eosine, but Jodlbauer and Tappeiner (58) observed that if the eosine solution was neutralized after irradiation and before the addition of the paramecia, this effect was not produced. The latter investigators claimed that the Ledoux-Lebard effect was due to the formation of acids which brought about the killing and lysis of the protozoa. They do not appear to have considered that some of the photodynamic phenomena which they studied might have been produced, or at least greatly influenced, by the formation of acid in the same manner. Blum (6), working in very well buffered sodium-phosphate solutions, was able to show that hemolysis of red blood cells could be produced by previously irradiated fluorescein dyes. These experiments, not being subject to the criticism offered by Jodlbauer and Tappeiner to Ledoux-Lebard's, seem to eliminate the necessity of assuming that cell and sensitizer must be irradiated together. Other observations have been recorded of the effects of previously irradiated dyes. Sacharoff and Sachs (98) found that previously irradiated "Indigosalz" produced hemolysis. Fabre and Simonnet (26) found that lecithin irradiated together with hematoporphyrin by light from a mercury vapor lamp may subsequently produce hemolysis in the dark. Moore (86) found that previously irradiated eosine in sea water was lethal to sea urchin's eggs. Since, when the eggs and the dye are irradiated together, the eggs are cytolyzed, Moore assumed two separate processes for killing and cytolysis. He suggested that a toxic substance is formed when the dye is irradiated and that this toxic substance produces cytolysis when it is further irradiated after having penetrated into the cell. Since lysis has actually been produced by previously exposed dyes in the case of red blood cells (6), and of paramecia (70), it seems probable that Moore's killing effect simply represents a less

degree of the same process which produces lysis. This is in accordance with the writer's finding (6) that hemolysis occurs more readily when the cells are irradiated together with the dye than when the dye is previously irradiated.

Although investigators have repeatedly failed to show photodynamic phenomena with previously irradiated solutions (36), (37), (38), (90), (96), the failures are undoubtedly due to experimental difficulties, certain of which have been discussed by Blum (6) (7).

Tappeiner's hypothesis assumes an interference with normal metabolic processes in the cell, and Metzner (82) suggests that the phototropic responses which he describes may be due to a shift in normal oxidations. However, cyanide does not inhibit photodynamic action (77), (17), (86), (4), (8), although it inhibits the action of all oxidative enzymes with the exception of the aerobic dehydrases.[6] This indicates that biological oxidations are not concerned in the photodynamic phenomena. The early hypothesis of Straub (105), which states that the photodynamic effects are due to direct oxidation of cell constituents, seems still the most satisfactory explanation and is completely in accord with photochemical findings. Numerous investigators have suggested that the proteins comprise the site of oxidative attack in the cell. Schmidt and Norman (101) found that hemolysis by eosine and sunlight could be prevented by the presence of tyrosine and tryptophane, and proteins containing these amino acids, but not by other amino acids or by gelatine which contains neither tyrosine nor tryptophane. They suggest that these easily oxidizable amino acids in the protein molecule are the point of attack in the cell. Harris (35) found that a large number of proteins all took up O_2 on exposure to light from the quartz mercury arc with the exception of gelatine; correspondingly, tyrosine and tryptophane were the only amino acids which were found to take up O_2. He found (34) that the absorption of O_2 was increased 30 to 40 fold by the addition of hematoporphyrin as a sensitizer. It might be expected that tyrosine and tryptophane being among the most readily oxidizable of the amino acids would be likewise the most easily attacked in this oxidative process. Carter (12) found that ring compounds containing a hydroxy or amino group were the only ones among a large number examined which were susceptible to oxidation by sensitizers and light. Such substances are, of course, known to be very readily oxidizable. This evidence indicates that the oxidation of easily oxidizable substances such as tyrosine and tryptophane is possible in the cells. It does not

[6] The nomenclature is that of Dixon (18).

prove that these amino acids constitute the point of attack or that other readily oxidizable substances, e.g., the sterols, might not be oxidized first.

It is of interest to note here that Harris' finding (35) that oxygen is absorbed by tyrosine, tryptophane, and proteins containing these amino acids, cannot be accepted as evidence that the oxidation of these compounds is the major process involved in the destructive action of ultra-violet light on living cells, since the latter effects may be produced in the absence of oxygen (see p. 29).

Howell has shown (49) that fibrinogen exposed to light with hematoporphyrin or eosine loses its ability to be coagulated by thrombin or by heat. According to Howell this represents some change in solubility of the fibrinogen and is the basis for the prevention of coagulation of blood by photodynamic substances and light.

TYPES OF PHOTODYNAMIC EFFECT. It would be impossible to discuss in detail the many instances of photodynamic action which have been reported, and the following brief treatment will be limited to a certain number of examples, other than those which have been mentioned above. A more or less arbitrary distinction may be made between the effects on those smaller organisms in which visible light may penetrate throughout the whole, or at least a great part of the organism, and those more complex forms in which the primary effects are limited to a very small part of the organism comprised by the surface layers.

Effects on smaller organisms. Numerous examples of the destructive effects on protozoa have been mentioned already. Many other small invertebrates are killed in a like manner, including coelenterates, nematodes, annelids, and echinoderm eggs or larvae (90), (92), (32). In the plant kingdom the destructive effects on various fungi have been studied, particularly among the bacteria (23), (58), (59). Ward (117) describes an interesting case of the production of a substance by *Bacillus influenzae* which brings about hemolysis of red blood cells when irradiated. This is probably a case of photodynamic action. Gicklhorn (32) found that various algae and the shoots or leaves of higher plants were injured by sensitizers and light. Among other effects, Gicklhorn found that the streaming movement of the protoplasm of *Nitella* was at first increased by light when a sensitizer was present, but with continued irradiation subsequently slowed down or ceased entirely. Tappeiner (110) cites numerous interesting examples of photodynamic action which are mentioned in the older literature.

A very interesting phenomenon appears in the induction of tropisms in forms which are not ordinarily phototropic. Metzner (81), (82)

found that strains of *Paramecium caudatum* and other one celled organisms which do not respond to light under normal conditions may be rendered positively or negatively phototropic by sensitization with dyes under proper conditions of illumination; he also demonstrated (83) induced tropisms in plant roots.

Effects on isolated tissues and organs of higher animals. The hemolysis of red blood cells has already been discussed above. Other components of blood are also affected; leucocytes cease their amoeboid movements and may undergo lysis (99), and the coagulating power of plasma may be destroyed (49), (4). Numerous investigators have described the production of arythmias in cold blooded hearts (2), (114), (118), (96), (104). Supniewski (107) using perfused mammalian hearts found that constriction of the coronary arteries occurred followed by rapid decrease in activity of the heart. Campbell and Hill (10) produced stasis in the capillaries of frog's mesentary by the action of light and hematoporphyrin or eosine.

Visible light may act as a stimulating agent to elicit responses in excitable tissues when a sensitizer is present. Hertel (48) found that the atropinized retractor of the esophagus of *Sipunculus niger* could be stimulated by light when injected with eosine. Adler (1) and Kolm and Pick (67) found that the smooth muscle of frogs could be stimulated photodynamically, and Supniewski (107) found that the smooth muscle of various organs of frogs and mammals both excised and in situ could be made to contract when irradiated with visible light after sensitization with hematoporphyrin.

Supniewski (107) and Lippay (73) found that frog skeletal muscle, when sensitized, responds to visible light. Lippay (73) found two types of contraction: *a*, a rapid contraction similar to the muscle twitch, and *b*, a slow prolonged contraction. The first type of contraction did not occur in curarized or denervated muscle, and he concludes that this type is due to stimulation of nerve or nerve endings, whereas the latter type of slow contraction is due to direct stimulation of the muscle. He found (74) a progressive irreversible reduction in irritability during the action of the light, and Lippay and Wechsler (75) found that sensitized muscles rendered inexcitable by treatment with isotonic cane sugar solution, potassium chloride, or ethyl alcohol still responded to light with the prolonged type of contraction. Thus it seems probable that this type of contraction is not related to the normal physiological responses of muscles. The similarity of these effects to chemical stimulation, e.g., by sodium citrate is obvious.

Lippay (73) could not demonstrate the photodynamic stimulation of frog's nerve. However, Lillie (72) was able to stimulate nerve sensitized with eosine by means of light from an incandescent lamp. The writer has been able to verify these latter observations. It seems probable that all the above types of stimulation are due to injury to the tissues and the analogy to chemical stimulation seems apparent. It seems probable that the oxidation of certain constituents upsets a balance in the system resulting in a response. This is in a way comparable to the upset of the ionic balance by the calcium precipitants.

Effects on larger animals. In whole vertebrate animals, as might be expected, photodynamic effects are more complex and irregular. Obviously, in the complex organism the only part which can be directly affected is the outer surface, since the penetration of light is limited to a relatively thin outer layer. Thus, in mammals the primary effects are found in the skin, but may be accompanied by marked secondary effects in distant organs, e.g., the circulatory, respiratory, and central nervous systems. The problem is complicated in vertebrates by the fact that the symptoms vary with conditions of irradiation and dosage with sensitizer. Hausmann (39, p. 47–9) describes the following groups of symptoms for white mice sensitized with hematoporphyrin under different conditions: *a*, Under extreme conditions of irradiation or dosage the animal may go into coma in a very few moments and die shortly after. *b*, Under less severe conditions an acute form occurs in which the animal scratches vigorously at first and waltzes about, in a few minutes the ears become inflamed and the animal blinks the eyelids and attempts to avoid the light; shortly he becomes weak and dyspneic; in some cases agonal tetany is observed; death may occur in a few hours. *c*, In subacute conditions, severe edema of the skin appears. *d*, With still less severe conditions the ears become necrotic and may slough off completely and the hair may fall out in a ring shaped area around the eyes. Various investigators have described effects which may correspond to one or the other of the above conditions. It seems obvious that the preliminary motor excitation in acute cases is due to sensory stimulation resulting from skin irritation, but that this is not the cause of death has been shown by Awoki (3) who found that white mice sensitized with hematoporphyrin and narcotized with urethane did not exhibit the usual dancing movements but died, nevertheless, in the same time as unnarcotized animals when exposed to light. Pfeiffer (93) describes symptoms and post-mortem findings in some detail. He also describes experiments on parabiotic white rats in which only one animal

was irradiated; the parabiotic mate died subsequent to the irradiation and usually before the irradiated animal. The findings at autopsy were the same in both animals. Rask and Howell (96) after an experimental analysis of hematoporphyrin sensitization in dogs conclude that death is caused by vascular collapse due to paralysis of cutaneous circulation but do not assign a definite cause. Pfeiffer (93) found marked changes in temperature during and following irradiation but Stammers (104) found no change. Kichiya (65) has given an interesting analysis of the chemical changes in the blood of rabbits sensitized to light by hematoporphyrin. Macht and Teagraden (78) found interesting changes in the maze running ability of rats when injected with quinine or quinidine and subjected to ultra-violet radiation.

We also find effects outside the laboratory which are probably due to the same mechanisms involved in the effects discussed above. It has been known for a long time that animals fed exclusively on buckwheat may develop serious symptoms (*fagopyrismus*) which occur only when the animals are exposed to sunlight. The animals go crazy and dance about, probably because of sensory stimulation from skin irritation. They develop severe skin eruptions, and the results are often fatal. Only white animals or animals having white spots are affected, and in spotted animals the skin lesions are confined to the white areas. This is undoubtedly due to the fact that the skin of the dark areas is protected by the absorption of the light by the pigmented hair. The symptoms of this disease are similar to those described above and there can be no doubt that this is a case of photodynamic action, the sensitization being due to an ingested substance from the buckwheat which is carried to the skin by the blood stream. Sheard et al. (102) give a very interesting analysis of this condition.

At the present time there is a problem of considerable economic importance in parts of the western United States in the matter of the poisoning of range animals by *Hypericum punctatum*, or St. Johnswort. This weed brings about symptoms similar to those caused by buckwheat. The poisoning by another species of this weed, *Hypericum crispum*, is supposedly responsible for the fact that only black sheep are raised in certain parts of Italy. It is stated that in Tunis, where this weed abounds, the Arabs often stain their white horses with henna or tobacco to protect them from light; for an interesting historical discussion see March and Clawson (80). A fluorescent extract from flowers of *Hypericum* acts photodynamically on erythrocytes according to Hausmann and Zaribnicky (46), and Joannides (50) sensitized rats to light

by injections of an extract of the whole plant. Hausmann (39) discusses other conditions in domestic animals which may be attributed to photodynamic action.

The possible relation of hematoporphyrin and other porphyrins abnormally present in the blood stream to light sensitization in the human can only be mentioned here. For a discussion of these conditions, the sensitization to light by drugs, and other possible relationships of these problems to the clinic, the reader is referred to Hausmann (39) or Hausmann and Haxthausen (40), or Lipschitz (76).

The destruction of numerous substances of biological interest, e.g., toxins and enzymes, has been reported but cannot be discussed at length in this article, it may be assumed that these represent photosensitized oxidations by molecular oxygen.

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THE REVOLUTION IN MUSCLE PHYSIOLOGY[1]

A. V. HILL

University of London, England

The revolution to which this paper refers broke out on the last day of December, 1926, when Eggleton and Eggleton (1) sent to the Biochemical Journal a paper describing "phosphagen," a labile form of organic phosphate in muscle. In 4½ years their discovery has changed our outlook on the relation between the chemistry and the energy exchanges of muscle to an extent that many do not fully realize. The new standpoint is now more or less stabilized: no doubt in a few years further discoveries will lead to further drastic change: at the moment, however, it may be profitable to make a general survey of the new position, and of the road by which it was reached.

There is no time now, nor is there need since most physiologists are so well acquainted with it, to discuss the older standpoint, in which the energy derived from lactic acid formation and removal occupied so prominent a place. I would, however, urge that those who write, or work, upon this subject should not imagine, because drastic changes have proved necessary in our view of the relation between lactic acid and the thermodynamics of muscle, that therefore most of the older results on which this view was based are wrong. No theory of muscular contraction had been invented: only a picture of the way in which energy and chemical exchanges, particularly of carbohydrate and lactic acid, are related. Much indeed of the older view is really experimental fact, and fortunately—how could science advance otherwise?—it is possible to make perfectly good experiments, even when one's theory is incomplete. This word of caution is not unnecessary: for example, in a recent paper on the mechanical efficiency of the human body, Simonson and Hebestreit (2) remark that what they are kind enough to call "die Hillschen Vorstellungen über den isolierten Muskeln" have been completely shattered by recent research, and they conclude that the bottom has thereby been knocked out of all the applications of the physiology

[1] A paper read to Section I of the British Association for the Advancement of Science on Friday September 25, 1931.

of the isolated muscle to the case of muscular exercise in man. I am ready, as you will see, to bear my share of the blame for an imperfect theory; as a matter of fact, however, so long as oxidative recovery follows activity, most of these applications to man are unaffected. It matters little to the oxygen debt after a bout of exercise whether we attribute it to "lactic acid formation" alone, or add "and phosphagen breakdown." It makes no difference to the mechanical efficiency whether the energy for contraction is derived chiefly from phosphagen, or chiefly from lactic acid. This is because the new facts in man were discovered by experiments conceived on the basis of the older facts in isolated muscle: they do not depend on any particular theory.

For several years there had been signs of the coming revolution:

a. In 1920 Hartree and I (3) pointed out that in stimulated muscles from which oxygen had been carefully excluded a small delayed heat production occurs during the three minutes following contraction. Every attempt—and we made many because nobody could see what it meant—to get rid of the delayed anaerobic heat failed,[2] until at last it is recognized as the sign of a reaction, recently discovered, by which phosphagen is restored anaerobically at the expense of energy liberated by delayed lactic acid formation.[3] It is a pity that Hartree and I did not insist more strongly on the pertinence of the fact we had found, but yielded to the scepticism of our friends and did our best, quite unsuccessfully, to explain it away. Indeed I should bear the blame myself, for Hartree was always very uncompromising on the subject!

b. In 1922 Embden (5) had found that phosphate diffuses far more rapidly into Ringer's fluid from a fatigued than from a resting muscle. Unfortunately, for he might have discovered phosphagen, he did not attribute this to the new appearance of inorganic phosphate inside the muscle, but to a change of permeability of its surface.

c. In 1924 Embden (6) (7) and his colleagues first reported experiments which claimed to prove that a considerable fraction of the lactic acid set free as the result of tetanic stimulation may appear after contraction is over. Unluckily, for various reasons to which I will refer later, this claim was not accepted, although it proved ultimately to be right. According to Embden the immediate energy for anaerobic muscular work was derived from some unknown colloidal chemical reaction: actually it is derived from phosphagen breakdown.

[2] For further references see Blaschko (4), Hartree and Hill (34).

[3] See Lundsgaard (26).

d. In 1925 Teigs (8), in Australia, had found that creatine diffuses far more rapidly from a fatigued muscle than from a fresh one, and that this excess diffusion is stopped by oxygen. He, like Embden, had nearly discovered phosphagen, but unfortunately the clue was not followed up. Teigs moreover claimed to show that in muscular activity not only acid but alkali is produced, the latter being in the form of a more alkaline creatine. He was remarkably near the truth.

e. In 1926 Hoet and Marks (9) discovered that muscles rendered glycogen-free by previous administration of insulin and thyroid may pass into a peculiar form of rigor, without lactic acid formation or increase of hydrogen ion concentration. The inorganic phosphate of the blood was found to rise. Here was foreshadowed the behaviour of muscles poisoned with iodoacetic acid, in which, as Lundsgaard showed later, contraction and contracture may occur without any lactic acid change.[4]

f. Finally there had always been a gap, never satisfactorily bridged, between *1*, the heat set free when 1 gram of lactic acid is liberated in normal intact muscle, and *2*, the heat accompanying the formation of 1 gram of lactic acid in minced muscle to which glyclogen and phosphate had been added. The difference was partly made up by the heat of reaction of lactic acid with the alkali-protein of the intact muscle; but Meyerhof had always maintained that something was still missing ((11) p. 222).

It had long been known that phosphorus compounds must have some special part to play in muscular contraction. Creatine, moreover, was known to be present in large amount, but nobody had suggested a function for it, or the manner of its combination. There still are substances, muscle hemoglobin, carnosine, even potassium, present specifically in muscle in relatively large quantities, but as yet no rôle has been found for them. As regards the phosphorus compounds, till phosphagen was discovered it seemed most likely that these were intermediaries in the breakdown of other substances: one tended therefore to disregard them in calculations of the energy exchange, and to concentrate on the initial and final substances of which we knew, glycogen and lactic acid. Had the so called "inorganic" phosphate really been inorganic, had the "organic" phosphate been chiefly a hexose phosphoric ester and the source of lactic acid (as the name "lactacidogen" implied), then one were justi-

[4] The first sign of the coming revolution, as Dr. H. H. Dale pointed out to me, was visible in 1877 when Claude Bernard (10) in his "Leçons sur le Diabète" stated that acidity is not the essential cause of rigor but only its usual accompaniment.

fied in regarding the phosphorus compounds as intermediaries, as not affecting the total energy exchange. We were, in fact, all wrong. The delayed anaerobic heat could not be disregarded, and several important things occurred, endothermic as well as exothermic, even in an oxygen-free muscle, long after contraction and relaxation were complete. The "inorganic" phosphate was chiefly a creatine-phosphoric ester, which broke down rapidly into its constituents when the muscle was stimulated or injured. The "organic" phosphate was not mainly a hexose ester, it was not the source of lactic acid, but was largely adenyl-pyro-phosphoric acid. I wonder whether we are still failing to see something which in ten years will seem obvious?

The Eggletons had shown, at the end of 1926, that when a muscle is excited phosphagen breaks down, and in the presence of oxygen is rather rapidly restored. The amount of phosphagen is greater in quick muscles than in slow (12), though recent work proves it to have the same rôle: the muscles being slower, there is more time for the phosphagen to be restored anaerobically, so the amount of it can be less. Its breakdown is greater, relative to lactic acid formation, in the earlier than in the later stages of activity and fatigue. Shortly after, and independently of, the Eggletons' publication, Fiske and Subbarow (13) reported the same discovery, and showed, in addition, that phosphagen is a compound of creatine and phosphoric acid: they concluded also as Teigs' work (8) suggested and as Meyerhof has since confirmed, that its breakdown affects the buffering power of the tissue.

At an early stage in 1927, just as Lundsgaard did later in 1930, P. Eggleton went to consult with Meyerhof, who at once got busy on the subject. By the autumn of 1927 Meyerhof (14) had shown that the heat of hydrolysis of creatine-phosphoric acid is considerable, and by the spring of 1928 Nachmansohn (15) had discovered the very pertinent fact that after activity a considerable part of the phosphagen broken down is restored rather rapidly, even in the complete absence of oxygen.

Here, however, was a dilemma. The breakdown of phosphagen—at least of creatine-phosphoric acid—leads to a considerable evolution of heat: its resynthesis, therefore, must lead to a considerable absorption. Hartree and I, however, had examined the heat production of muscles, during and after activity, in oxygen and in nitrogen, with the greatest care. There was no evidence, at any stage, of an absorption of heat ((16) p. 210). Often as "negative Wärmeschwankungen" had been reported in the past they had invariably been traced to experimental error. We could not admit the possibility of heat absorption following

activity: rather, we had again to point out the existence of a small positive delayed anaerobic heat, at any rate after a tetanus. If, therefore, the resynthesis of phosphagen really occurred it must be accompanied by some strongly exothermic reaction. No such reaction had been suggested except delayed lactic acid formation, and this unfortunately Meyerhof and his colleagues—in spite of Embden—denied. We concluded ((17) p. 168), and Meyerhof was forced to conclude ((11) pp. 110, 220),—though only for a time—that the phosphagen was "unstabilized" in some way by stimulation, "re-stabilized" in recovery, but never actually broke down.

Let us return to lactic acid. As early as 1924 Embden and his colleagues (6) (7) had claimed to show that a considerable part of the lactic acid set free as a result of a 5 to 10 second tetanus may appear after the contraction is over. Meyerhof and Lohmann (18), (19), in 1925 and 1926, failed to confirm this claim, and believed they had shown it to be due to excessive direct stimulation. The delayed anaerobic heat was not nearly large enough, in any case, to cover the lactic acid formation stated by Embden to occur: and since phosphagen, and its breakdown and endothermic restoration, had not then been discovered, there was no way of fitting a delayed lactic acid formation of the required magnitude into the scheme. It was easier, therefore, to agree with Meyerhof that the delayed lactic acid was due to experimental error. Embden, however, returned to the charge (20), and (while admitting that he might have over-stimulated his muscles in previous experiments) described new experiments designed to establish the same result. Most unfortunately, for his conclusion was actually correct, his argument and his experiments did not prove what they were intended to prove: a subtle point in logic had escaped his attention (see (21) p. 180). So again, this time for a better reason, his results were not accepted. He laughs best, however, who laughs last, and lactic acid is, in fact, produced, after contraction and relaxation are over!

So, however, for a time the matter remained. Phosphagen was discovered, together with its partial resynthesis anaerobically after stimulation, and the heat production of its breakdown. Clearly a delayed lactic acid formation now became possible, since the absence of sufficient delayed anaerobic heat could be explained by the simultaneous occurrence of the endothermic restoration of phosphagen. The experiments, however, which claimed to establish it had been criticised, so the dilemma appeared to remain (see (11) p. 110). In the meantime, in 1928, Lohmann (22) proved that a considerable part of the supposed organic

phosphate (other than phosphagen) was really pyrophosphate, which later was shown (23) to be linked with the adenylic acid first discovered in muscle by Embden and Zimmermann (24): this compound has recently been found to be part of the co-ferment system in the hydrolysis of carbohydrate, and also to yield ammonia when it breaks down to inosinic acid and orthophosphate (see (11) pp. 88, 173). Much work, also, was done by Meyerhof and his colleagues on the titration curves of the various phosphorus compounds present in muscle and of their breakdown products; until, early in 1930, Meyerhof (25) made the very significant discovery that in a stimulated muscle, during the early stages of activity, a change occurs in the CO_2-binding capacity of muscle which could only be explained if phosphagen really *was* broken down into creatine and inorganic phosphate. A muscle might even combine with more CO_2 as the result of activity, which could not be the case if lactic acid were produced without any compensatory buffer change. Apparently phosphagen actually *did* break down: but why, in that case, was there no absorption of heat when its resynthesis occurred after activity?

At this critical stage, early in 1930, the first paper by E. Lundsgaard of Copenhagen appeared, in which it was shown that muscles poisoned with iodo-acetic acid may contract, apparently perfectly well for a time, without any lactic acid formation at all, only with the breakdown of phosphagen. Lundsgaard's work, admirable in expression and execution and in the way the initial clue was followed up, is contained in six papers (26), published in the Biochemische Zeitschrift in 1930 and 1931. After about 100 twitches (in place of the 400 which a normal muscle can give before exhaustion) a curious form of contracture sets in, and the muscle ceases to be excitable, or to be capable of recovery, even in oxygen. By this stage all, or nearly all, the phosphagen is broken down. In crustacean muscle, where phosphagen is arginine-, instead of creatine-, phosphoric acid the same thing happens. Apparently phosphagen breakdown can supply all the energy for a perfectly normal contraction. It is interesting now to re-read the paper by Hoet and Marks (9) in view of Lundsgaard's results. It was shown, moreover, by Fischer (27), and confirmed by others, that the ratio Tl/H in a muscle twitch (where T is isometric tension developed, l is muscle length and H is initial heat) is the same in an iodo-acetic acid poisoned muscle as in a normal one: and by Hartree (28) that the distribution of heat in the initial phases of contraction also is quite unaffected by preventing the formation of lactic acid. Lundsgaard further found that in a poi-

soned muscle there is no anaerobic resynthesis of phosphagen after contraction: if lactic acid cannot be produced phosphagen cannot be resynthesized.

Lundsgaard immediately proposed the hypothesis that "phosphagen is the substance directly supplying the energy for contraction, while lactic acid formation in the normal muscle continually provides the energy for its resynthesis." In that case, however, since part of the resynthesis is delayed long after the contraction, part at least of the lactic acid formation must be delayed too. It became reasonable, and indeed necessary, to believe in the delayed formation of lactic acid, and Meyerhof and Lundsgaard on the one hand (Lundsgaard was now for a time in Meyerhof's laboratory) and Lehnartz on the other in Embden's laboratory, independently and with redoubled efforts, looked for it again and found it (26) (29) (30): this time by methods and argument beyond reproach! By the late autumn of 1930 it was certain at last that a considerable part, if not all, of the lactic acid set free as the result of stimulation, appears after the contraction is over, during the period in which the phosphagen is restored. The revolution was complete. One major question remained—and remains—unanswered—why can only a part of the phosphagen be restored at the expense of lactic acid, why does a considerable fraction still wait for oxidative resynthesis?

Such is a short history of one of the most interesting, and to a participant one of the most exciting, developments of recent physiology. From 1907 when Fletcher and Hopkins (31) first put the study of lactic acid in muscle on an exact basis, to the final stage in 1931, progress has depended upon the coöperation of many workers in various different countries. There has been much friendly discussion hidden behind the published papers, and a certain amount of open controversy. We have all been right sometimes and we have all been wrong often. Perhaps if there had been more discussion and less controversy the end might have been sooner!

Let me summarise, therefore, the main facts in relation to lactic acid and phosphagen, omitting the other factors which seem less pertinent at present, at least in a discussion of the energy exchanges. In the oxygen-free muscle:

1. On stimulation phosphagen breaks down into creatine[5] and phosphate, with a liberation of 120 calories per 1 gram of H_3PO_4: this is the primary change by which energy is set free.

[5] In crustacean muscle into arginine and phosphate.

2. The acid combining power of the tissue is increased, more at greater, less at lower hydrogen ion concentration, as a result of this breakdown.

3. *a*. During contraction and relaxation, and *b*, in the next few minutes, lactic acid is set free. In a 5 second tetanic contraction of a frog's muscle at 20°C. not more than half the lactic acid appears during *a*, not less than half during *b*: in a twitch, possibly none in *a*, all in *b*. When owing to the accompanying phosphagen change, there is no alteration of pH in the whole process, the heat liberated is about 200 calories per 1 gram of lactic acid. When, in a more fatigued muscle, the pH decreases as the result of contraction, the heat liberated includes that due to the repression of the ionization of alkali-protein, which in the extreme case may amount to 100 calories per 1 gram of lactic acid.

4. The restoration of phosphagen in anaerobic recovery is an endothermic process, masked, however, by the exothermic production of lactic acid. The energy liberated by delayed lactic acid formation allows the resynthesis of phosphagen; this, however, does not occur if lactic acid formation is impossible, as in a carbohydrate-free muscle or in one poisoned with iodo-acetic acid.

5. The delayed anaerobic heat is the resultant of *a*, the exothermic reactions of lactic acid formation and protein unionization, and *b*, the endothermic reactions of phosphagen resynthesis and protein ionization.

6. The heat in a series of twitches can be accounted for closely by the resultant phosphagen breakdown and the total lactic acid formation.

7. If the lactic acid formed in a slow series of twitches is continually removed by diffusion, the muscle can go on working far longer than if the lactic acid be allowed to accumulate (32). Presumably in the later stages, when there is little phosphagen left, the anaerobic resynthesis after contraction is very nearly complete.

8. In a muscle poisoned with iodo-acetic acid all the energy for contraction is derived from phosphagen breakdown. In the normal muscle the same is probably true, so far as concerns the energy in contraction alone, as distinguished from aerobic or anaerobic recovery. There is nothing inconsistent in regarding the *whole* of the lactic acid set free as being concerned with the resynthesis of phosphagen broken down during activity (see Appendix).

In a muscle supplied with oxygen there seems to be no doubt that

the same events occur as in an oxygen-free muscle, followed or accompanied by oxidative recovery. Only part of the phosphagen broken down is restored anaerobically at the expense of delayed lactic acid formation: in a muscle with oxygen the rest is reinstated rather rapidly, as the Eggletons showed (12), before the main part of the lactic acid is dealt with. The recovery heat represents the excess of energy liberated by oxidation, over and above that required for phosphagen and glycogen resynthesis. The ratio of total heat in oxygen to total heat in nitrogen is almost exactly the same, 1, in a single short contraction in which probably most of the anaerobic heat is derived from phosphagen breakdown (2.0 see (34) p. 135), and 2, in a long and rapid series of twitches in which much of the anaerobic heat is derived from lactic acid (2.07, see (33) p. 190). In other words, supposing the oxidation to be the same in either case, and the mechanical efficiency of the initial process to be the same, the total oxygen per unit of work performed should also be the same, whether that work be great or small. This conclusion, interesting enough in itself in connection with the mechanism of recovery, is fundamental to the case of human muscular exercise: for it shows that the results of experiments relating mechanical work to oxygen used are unaffected (except in the manner of their expression) by the coming of phosphagen and the partial eclipse of lactic acid.

I referred earlier to the paper of Simonson and Hebestreit (2). Armed with their conviction that the revolution of which I have spoken had destroyed the basis on which the newer physiology of human muscular exercise had been built, they proceeded to make experiments on the efficiency of muscular work which—were the results correct—would indeed have brought the edifice tumbling in ruins! They claim to have shown that the mechanical efficiency of a man continually and largely increases as the spell of work is lengthened. In one case the efficiency was twice as great in a 6 minute as in a 1 minute spell, 80 per cent greater in a 3 minute than in a 1 minute. In the third minute a remarkable efficiency indeed must have been attained! Even assuming the second minute to have been only as efficient as the third, both were three times as efficient as the first: assuming a gradual change of efficiency, that in the third minute must have been four or five times as great.

There is no sign of such an effect in the isolated muscle, as Cattell and Lundsgaard (35) have shown: more convincing, however, in properly controlled experiments there is no sign of it in man. Crowden (36) has recently completed a research on analogous lines with a bicycle

ergometer, with which it is possible to ensure that the movement studied is of constant type and made not only at a constant frequency but at a *constant speed*: speed is well known to have a large effect on efficiency. Crowden's subject undertook his task in two ways: in the first, he pedalled for 25 seconds and rested for 3 minutes, repeating the process ten times: in the second, he pedalled continuously at the same rate and against the same load for 250 seconds. In the 3 minute interval after each 25 seconds of work recovery must have been nearly complete: after 250 seconds of work there was a considerable oxygen debt. The total amount of work was the same in each case. In a number of experiments the mean efficiency of the short spell intermittent work was 19.9 per cent, of the long spell continuous work 18.2 per cent, practically identical values. The difference, if it be significant at all, is in the opposite direction from Simonson and Hebestreit's. Had the effect they described appeared, the efficiency of the long spell continuous work would have been 30, 40 or 50 per cent. Their results cannot possibly be explained by the new chemistry of muscle. They must have changed the character of their movements between the shorter and the longer spells, settling down to a more efficient movement as time went on.

Crowden made further experiments on human muscular work, again on a bicycle ergometer, under what may be described as aerobic and anaerobic conditions respectively. The "aerobic" work was performed intermittently, 10 seconds of rapid pedalling against a heavy load followed by 3 minutes of rest, the whole repeated 6 times: the "anaerobic" work was performed continuously, 60 seconds at the same rate against the same load. The "aerobic" work was performed with the muscles always practically in the fresh condition: the "anaerobic" work finished with the muscles practically exhausted, with a high oxygen debt. The efficiencies were low because of the high speed. The mean values were:

"Aerobic" work	13.5 per cent
"Anaerobic" work	13.7 per cent

The identity of the two values confirms the conclusion above. After the short intervals of "aerobic" work the oxygen must have been used largely in restoring phosphagen; after the long interval of "anaerobic" work the oxygen must have been employed mainly in removing lactic acid. The efficiency, however, was the same.

There are, of course, other obvious ways in which the new knowledge of the isolated muscle can be tested and applied in the case of man. For

example, in view of what is known of phosphagen, a further study of phosphate and creatine in blood, before and after sudden exhausting exercise, is obviously needed. In 1930 Margaria (37) showed that the osmotic pressure of human blood may rise by 10 per cent as the result of only 1 minute of severe exertion: no doubt this is due mainly to withdrawal of water from it by the muscles: it is unlikely, however, that some of the products of muscular activity will not pass out: indeed many of them are known to do so. By such means the old knowledge must be amplified by the new. I admit that a page or two of theory in the text books will have to be rewritten: most, however, of the facts can remain. And in casting out the old Vorstellungen, whosesoever they are, let us treat them kindly, for they deserve our gratitude for leading us, perhaps by a devious road and over unexpected obstacles, to the new knowledge.

APPENDIX. The following average results, taken from the most recent paper by Lundsgaard (26), are instructive.

Frog' gastrocnemii, 18° to 20°C., resting or 5 seconds maximal tetanus.

Mean of 22 muscles, resting:

Lactic acid 0.17 mgm. per 1 g.

Phosphagen (H_3PO_4) 2.00 mgm. per 1 g.

Mean of 11 muscles, stimulated:

A, killed in liquid air immediately at end of tetanus:

Lactic acid 0.36 mgm. per 1 g.

Phosphagen (H_3PO_4) 0.80 mgm. per 1 g.

B, killed in liquid air after 1 minute rest:

Lactic acid 0.58 mgm. per 1 g.

Phosphagen (H_3PO_4) 1.38 mgm. per 1 g.

Heat, assuming 200 calories per 1 gram of lactic acid, 120 calories per 1 gram H_3PO_4 for phosphagen:

A, 0.182

B, 0.156

There would appear therefore to be a negative delayed heat. We must take account however of the acid-base changes. When phosphagen is broken down in excess at first the muscle becomes more alkaline and proteins ionize, with an absorption of heat: the 0.182 is diminished. When phosphagen is restored later the muscle becomes less alkaline, and additional lactic acid adds to the effect: the proteins unionize, heat is given out and the 0.156 is increased. The net effect is that B becomes slightly greater than A, hence the delayed anaerobic heat.

We can calculate, moreover, (neglecting acid-base changes) that *during* the 5 second tetanus only 0.038 calorie out of 0.182 calorie is derived from lactic acid formation: the rest comes from phosphagen breakdown. It is natural to suppose that this small fraction represents "delayed" lactic acid formation which has occurred during the later part of the 5 seconds of the tetanus—i.e., during the restoration of a phosphagen set free in the earlier part.

Thus it is reasonable to regard the *whole* of the lactic acid set free as being concerned with the restoration of phosphagen broken down during activity. It is unfortunately impossible to make similar experiments on a single twitch.

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THE LIVING CELL AS AN OSMOTIC SYSTEM AND ITS PERMEABILITY TO WATER

BALDUIN LUCKÉ AND MORTON McCUTCHEON

The Laboratory of Pathology, School of Medicine, University of Pennsylvania, Philadelphia, Pa.

When two aqueous solutions of unequal osmotic pressure are separated by a membrane permeable to water but relatively impermeable to the solutes, water tends to diffuse from the solution in which its concentration is higher to that in which it is lower, until the difference in osmotic pressure is equalized. Such a system is known as an osmotic system.

We are here concerned with the questions: Is the exchange of water between the cell and its environment subject to the laws of osmosis and of diffusion? Are the changes in the volume of the cell, which can be brought about by changing the external concentration of its medium, governed by the laws of osmotic equilibrium? Can the rate at which such changes take place be predicted from the laws of diffusion?

The answer to these questions will be considered in the first and second section of this review. The third section will be devoted to an intimately related subject—the permeability of the cell to water, that is to say, the relative ease with which water enters or leaves the cell under the driving force of osmotic pressure.

That the living cell does, in fact, behave as an osmotic system, is the view held by the great majority of observers today. This view implies that the cell acts as if it possesses a semipermeable membrane; i.e., a protoplasmic layer, which, in general while permeable to water, is more or less impermeable to many dissolved substances; also, that transfer of water between the cell and an anisotonic medium depends chiefly on the difference in osmotic pressure between cell contents and medium, and follows the laws of osmosis and diffusion.

The contrary view, held by a minority, is that the cell possesses no semipermeable membrane and that swelling of cells in hypotonic solutions is due chiefly to imbibition of water by cell colloids.

The present review will not attempt to decide between these opposing views in the case of all types of cells. The writers are prepared to

admit that in certain cells, such as the storage cells of seeds, the transfer of water across the cell surface is a phenomenon probably too complicated to be explained solely by the simple laws of osmosis. With such material, however, tissues rather than cells are generally studied, and tissues contain intercellular structures which apparently possess properties different from those of the cells. In other words, the osmotic properties of tissues cannot justifiably be assumed to be identical with those of their component cells.

Nor are cells which have been forcibly isolated from tissues suitable objects for osmotic studies, unless it can be shown that such cells have not suffered injury. The properties of injured and dead cells are quite different from those of uninjured cells, as will be shown later.

What types of cell are, then, suitable material for osmotic studies? First, free cells such as blood cells and certain marine ova, especially if criteria for injury can be applied; second, tissue cells (chiefly of certain plants) in which the individual cells can be directly measured under the microscope; third, with reservations, certain whole muscles, whose properties appear to approximate those of their component cells.

It must be pointed out at the start that a cell cannot be an *ideal* osmometer. To be an ideal osmometer both cell and medium would have to behave as ideal solutions and the membrane must be perfectly semipermeable. Such a condition can obviously not be realized in practice. Our task is rather to determine whether cells *approximately* obey the osmotic laws; how good is the agreement between observed and theoretical volumes when cells are placed in anisotonic solutions? If this agreement should be found to be satisfactory, it would go far to justify the conclusion that cells of the types discussed do in fact behave as osmotic systems. It would indicate that factors other than osmotic forces are, at least under certain conditions, relatively unimportant in determining cell volumes.

I. The laws of osmotic equilibrium developed by van't Hoff (119), while strictly valid only for ideal solutions, are, in general, approximately obeyed by actual dilute solutions. The application of these laws to the living cell forms the subject of the first part of this review.

A. *Applicability of the Avogadro-van't Hoff law to cells.* The law of Avogadro as applied to dilute solutions may be stated, equal volumes of isosmotic solutions (of non-electrolytes) contain the same number of molecules at a given temperature. It follows that all equimolar solutions (of non-electrolytes) are isosmotic.

Solutions of electrolytes exert a higher osmotic pressure than solutions

of non-electrolytes of equal molar concentration. The relation between the osmotic activity of solutions of these two classes of substances may be expressed by a factor, the isotonic coefficient of deVries, or van't Hoff's factor, which represents the number of times the osmotic pressure of an electrolyte solution is greater than an equimolar solution of a non-electrolyte.

The technical difficulties and, more particularly, the difficulty of preparing truly semipermeable membranes, have so far permitted direct measurement of osmotic pressure for solutions of only a few substances. However the inter-relation existing between the various colligative properties of solutions—osmotic pressure, freezing point, boiling point, and vapor pressure—permits calculation of the value of osmotic pressure which theoretically would be obtained in an ideal osmometer (30).

We are here concerned with the question: does Avogadro's law apply to living cells? 1. If the living cell be an approximately perfect osmometer, then, by Avogadro's law, its volume should be the same in all solutions having the same osmotic pressure. 2. If the concentrations are known in which two solutions are isotonic with the cell, we should be able to calculate the molecular weight of the solute of one when that of the other is known. 3. Having determined the concentration of a solution isotonic with the cell it should be possible to calculate the concentrations of other substances also isotonic with the cell, from the molecular weights. 4. And, finally, it should be possible to measure with cells the relative osmotic pressures of solutions; i.e., determine their isotonic coefficients.

Values obtained by using cells as osmometers should agree with those found by means of non-living systems.

1. *Experiments with plant cells.*[1] A typical mature plant cell may be considered, semi-schematically, as consisting of a solution of various electrolytes and non-electrolytes (the cell sap) enclosed in a semi-permeable membrane, the extremely thin protoplasmic layer (protoplast). The latter forms a closed sac lying against the semi-rigid cellulose wall (the cell wall), which in most plants is permeable both to water and solutes (108). The pressure exerted by the sap keeps the cell wall under a certain tension to which is due the normal state of rigidity (turgor) of the cell.

When a plant cell is placed in pure water or in a solution hypotonic with respect to its contents, entry of water due to endosmosis is gener-

[1] For detailed discussion the excellent monographs of Livingston (74) and of Stiles (113) should be consulted.

ally prevented by the rigidity of the wall, though in certain cells entry does take place, and as a consequence the wall is put under greater tension and may even rupture. When, on the other hand, a cell is placed in a hypertonic solution, exosmosis occurs, the sap diminishes in volume and the encircling protoplasmic layer retracts from the cell wall, which loses its tension. The phenomenon, discovered independently by Naegeli (92), (who introduced the term plasmolysis), and by Pringsheim (102) was utilized by de Vries (120, 121, 122) in his classical studies on the relative osmotic pressure of solutions.[2]

By placing cells of the same kind in solutions of graded concentration it is possible to find for each solution that particular concentration in which plasmolysis is barely perceptible ("incipient plasmolysis"). If the protoplasmic membrane is strictly semipermeable and the volume of the cell is governed solely by osmotic forces, all solutions isotonic with the same kind of cell must have the same osmotic pressure.

In practice, the relative osmotic pressure of solutions was expressed in terms of an isotonic standard solution, used as control in every experiment in order to make the determinations independent of any temporary condition of the cells. As such, deVries (122) used KNO_3 solution, so that the results could be expressed as "KNO_3 values" or as "isotonic coefficients." DeVries found that solutions of sucrose, dextrose and various other non-electrolytes effected the same volume changes of the protoplast, i.e., incipient plasmolysis, in equimolar solutions. But solutions of salts and other electrolytes were found to be isotonic in *lower* concentrations. Thus, for example, with certain cells, the following solutions were isotonic in the concentrations stated: 0.21 M. dextrose or sucrose, 0.13 M. KCl or KNO_3, 0.10 M. K_2SO_4, 0.08 M. K_3 citrate. DeVries expressed the ratio of the osmotic activity of solutions by the term "isotonic coefficient." The numerical value of the isotonic coefficient of KNO_3 was chosen as 3. (At the present time the osmotic activity of solutions is compared with that of cane sugar taken as unity) (4). The isotonic coefficient, i, of sucrose would therefore be $i:3 = 0.13:0.21$, that is, about 1.8. Or, in other words, a solution of sucrose would have only about two-thirds the osmotic pressure of a solution of KNO_3 of the same molarity. The methods devised by deVries and his experi-

[2] Details of various plasmolytic methods are to be found in the papers by de Vries (121), Fitting (27), Höfler (48, 49, 51) and others, as well as in the articles by Grafe (35), by Küster (66) in Abderhalden's Arbeitsmethoden, and by Pringsheim (103) in Peterfi's work. For a general discussion the monographs of Livingston and Stiles should be consulted.

mental determinations of the relative "water attracting power" of substances in solution were of fundamental importance for the later development of the theory of solution and of osmotic pressure (52).

The term isotonic coefficient, as now used, expresses the degree of deviation of electrolyte solutions from the "normal" activity of non-electrolyte solutions with respect to osmotic pressure, freezing point, etc. Or, in other words, the isotonic coefficients express the number of times the osmotic activity of a certain electrolyte solution is greater than that of a non-electrolyte in equimolar solution.[3]

a. If it be true that living cells are natural osmometers, and if the law of Avogadro-van't Hoff can be applied to cells, it should be possible to use cells as osmometers for the determination of the molecular weight of various compounds. This, in fact, was accomplished by deVries (122, 123), and his experiment furnishes brilliant support for the view that the law under discussion is obeyed by the cell. At the time of deVries' experiment three different values had been proposed for the molecular weight of the sugar raffinose, the formulae and molecular weights proposed being: $C_{12}\ H_{22}\ O_{11} + 3H_2O = 396$, $C_{18}\ H_{32}\ O_{16} + 5\ H_2O = 594$, and $C_{36}\ H_{64}\ O_{32} + 10\ H_2O = 1188$. Using as osmometers the cells of *Tradescantia discolor* he determined the concentrations of raffinose and of sucrose which at equilibrium brought about the same volume change in the cells, i.e., just caused the protoplast to retract from the cellulose wall. It was found that 5.957 per cent solution of raffinose produced the same volume change as a solution of sucrose containing 0.1

[3] In order to avoid misunderstanding it should be pointed out that the concentration of a solution isotonic with the cell in the state of incipient plasmolysis is not always a measure of the osmotic pressure of the cell in its normal turgid condition, even though its plasmatic membrane be strictly semi-permeable and no injury occur during the experiment. Since the cellulose wall of many plant cells is considerably distensible, the volume of the protoplasmic sac of the cell in the state of incipient plasmolysis may differ from that in the normal turgid condition. An example by Ursprung (117) makes this clear. A certain cell in its "normal" condition, when measured in paraffin oil, had a volume of 14122 (in arbitrary units). After being immersed in a solution of cane-sugar of sufficient concentration to produce incipient plasmolysis the volume was 13209. Upon being placed in pure water the volume increased to 14779; this condition was designated as "saturated." It follows that the osmotic concentration (osmotic value) of the plasmolyzing solution is constant only when the cell wall is not distensible. The terminology relating to the osmotic properties of cells in these several states, i.e., in the normal, at incipient plasmolysis, and in the saturated condition is somewhat confused. A full discussion of the terminology proposed by Ursprung and Blum (115, 116, 118) will be found in papers by Höfler (50), Stiles (112, 113), and Beck (5).

gram molecules per liter. These two isosmotic solutions should contain the same number of molecules, i.e., a 5.957 per cent solution of raffinose should be a 0.1 molecular solution. The molecular weight of raffinose therefore should be about 595.7. It is today accepted as 594.3.

b. Conversely, Overton (98) calculated from the molecular weights the concentration of various non-electrolytes which should produce the same degree of plasmolysis as a certain solution of sugar isotonic with the cell. With cells of *Spirogyra* he determined that a 6 per cent solution of cane sugar (molecular weight 342) would produce incipient plasmolysis; 2.63 per cent solution of arabinose (molecular weight 150) should produce the same volume change, since $\frac{6 \times 150}{342} = 2.63$. The concentration experimentally found was 2.7, a very good agreement indeed.

In the table are given the observed and calculated values of a number of isosmotic solutions determined by this method.

COMPOUND	MOLECULAR WEIGHT	ISOSMOTIC CONCENTRATION	
		Observed	Calculated
		per cent	*per cent*
Sucrose	342	6.0	
Manitol	182	3.5	3.19
Dextrose	180	3.3	3.16
Arabinose	150	2.7	2.63
Erythritol	122	2.3	2.14
Aspargin	132	2.5	2.32
Glycin	75	1.3	1.32

c. One of the strongest supports for the applicability of the Avogadro-van't Hoff law to cells is that the relative osmotic pressure of solutions (the factor i) as determined by plasmolytic methods is in close agreement with determinations made by such physical methods as depression of freezing point, electrical conductivity, etc. Many such determinations have been made by de Vries (122, 124) and others; the more recent ones of Fitting (28) are probably the most exact. He used the leaf-cells of *Tradescantia* (*Rhoeo*) *discolor* as osmometers, and by an improved plasmolytic method determined the isotonic coefficients for a number of salts. In the table given below some of his results are compared with values of i calculated from freezing point and conductivity measurements.

	VALUES OF i CALCULATED FROM		
	Plasmolytic method	Freezing point	Conductivity
KNO_3	1.69	1.78	1.829
KCl	1.74	1.84	1.86
KBr	1.74	1.84	1.87
$KClO_3$	1.73	1.75	1.81
K_2SO_4	2.27	2.36	2.38
$NaNO_3$	1.7	1.8	1.83
NaCl	1.71	1.84	1.83
$LiNO_3$	1.83	1.77	1.82
LiCl	1.8	1.85	1.81
$MgSO_4$	1.05	1.1	1.329
$Mg(NO_3)_2$	2.54	2.55	2.43
$Ca(NO_3)_2$	2.43	2.4	2.35
$Ba(NO_3)_2$	2.23	2.22	2.2
$BaCl_2$	2.42	2.46	2.414
$MgCl_2$	2.49	2.64	2.45
$Sr(NO_3)_2$	2 35	2.43	2.31
$SrCl_2$	2.49	2.55	2.46
$CaCl_2$	2.46	2.59	2.45

Unfortunately the solutions used for determination of depression of freezing point and for the plasmolysis experiments were weight-normal while for conductivity measurements volume-normal solutions were used. The error introduced is however not great. In the first group of the table are shown salts that penetrate slightly into the cell; the values of i from plasmolysis are therefore somewhat below the values obtained from physical measurements. In the second group of salts no penetration is demonstrable, and all the values are in good agreement. In the third group of salts the agreement is less good, although no penetration of salts could be demonstrated. The general agreement of the values, however, is evidence that the law of Avogadro-van't Hoff is obeyed by these cells.

2. *Applicability of the Avogadro-van't Hoff law to animal cells and tissues. Experiments with erythrocytes.* Probably no other kind of animal cell has been so extensively employed for investigation of osmotic phenomena as the red blood cell. The advantages of this cell for osmotic studies were early realized (for historical references see Overton (100), Gough (34)), and have very recently been pointed out again by Jacobs (56, 57, 58). These isolated cells are easily obtainable at any time and in any desired quantity, they have a relatively simple structure, they

can be kept in usable condition for a longer period of time than any other kind of cell, and unlike many other isolated cells erythrocytes show no seasonal variation. The mature mammalian erythrocyte, however, is a very unique kind of cell, if indeed it may be called a living cell at all, and because of its peculiar structure and function, its osmotic properties are perhaps more influenced by slight environmental changes than is the case with any other kind of cell. The effects of such environmental changes as change of pH, temperature, etc., have very recently been discussed by Jacobs (56, 57, 58) to whose papers reference should be made for details.

A further unique property of the erythrocyte is its peculiar permeability. In common with other cells the erythrocyte is freely permeable to a great variety of organic compounds whereas osmotic and electrical conductivity studies have demonstrated that it is impermeable to salts as such. However, unlike most other cells, the erythrocyte is permeable to many anions, so that an exchange of anions may take place between the cell and its surrounding medium. For example, it is well known that HCO_3^-, when its amount increases in the blood corpuscle, is exchanged for Cl^- from the plasma. For a discussion of the "differential permeability" of erythrocytes and of its peculiar sensitivity to certain environmental changes reference should be made to Hamburger (40), (older lit.), Höber (47), v. Tschermack (114), Bayliss (3), and Gellhorn (31), and particularly to the recent publications of Ege (16 to 20), Mond (91) and Jacobs (58). Failure on the part of numerous investigators in the field of osmotic phenomena to take into account the peculiar properties of erythrocytes has led to an astonishing diversity of results, and, indeed, to the denial by some (e.g., M. H. Fischer (25, 26)) that the cell is an osmotic system.

In erythrocytes, as in other cells, osmotic changes are always associated with volume changes. Two simple methods have been used for the study of such volume changes in the erythrocyte, namely, the hematocrit method and the method of hemolysis.

The hematocrit method was introduced by Hedin (41). A suspension of erythrocytes is introduced into a graduated tube of narrow bore which is then rotated in a centrifuge until the cells are closely packed together. By means of this method the total volume of all the cells in a given sample is obtained. The hematocrit tube of Hedin has been modified by Koeppe (61), Hamburger (38) and others. For technical details the original papers should be consulted, as well as the chapter by Höber (46) in Abderhalden's Handbuch; for recent critique see the paper by Ege (14).

The second method, that of hemolysis, first systematically used by Hamburger (37), is based on the observation that when the erythrocyte in swelling reaches a certain volume its hemoglobin escapes to such an extent as to render the cell almost colorless and hence invisible, while the surrounding medium becomes colored. This critical volume varies with the species of animal and probably also with individual erythrocytes.

The fact that the critical volume at which cells hemolyse may be affected by factors other than osmotic ones is not a serious disadvantage, since it is possible by proper controls to determine in any experiment whether osmotic factors alone are involved. For discussion of methods see Ponder (101), and particularly the recent paper by Jacobs (56).

We shall now give experimental evidence showing that the law of Avogadro-van't Hoff is applicable to erythrocytes.

a. Hamburger (37) first established the fact that the relative osmotic pressure of solutions may be determined by using the erythrocyte as osmometer. His method is based on the principle that there is a critical volume of the erythrocyte beyond which it loses hemoglobin. He found that this critical volume is the same in all solutions of the same osmotic pressure, no matter what the nature of the solution, provided that the substances in solution do not enter or injure the cell. In the table given below are compared the relative values of the osmotic pressure of various solutions obtained by this method, with the values obtained by deVries using the plant cell as an osmometer. The concentration is stated in per cent.

	HAMBURGER (ERYTHROCYTE)	DE VRIES (PLANT CELL)
KNO_3	1.0	1.01
NaCl	0.58	0.585
K_2SO_4	1.111	1.305
Sucrose	5.96	5.13
K Acetate	1.03	0.98
K Oxalate	1.225	1.245
$MgSO_4 + 7H_2O$	3.39	3.69
$MgSO_4$ anhyd	1.78	1.80
$CaCl_2$	0.823	0.832

The agreement between these two sets of values is remarkable. Determinations by physico-chemical methods have shown that the solutions in the concentrations stated above are in fact very nearly isosmotic (39). Experiments of this kind show, therefore, that in all isosmotic solutions the erythrocyte has the same volume, i.e., that the law of Avogadro-van't Hoff applies.

b. Equally convincing are experiments of the type performed by Koeppe (61, 62), Grynns (36), and by Hedin (41, 42), with the hematocrit. It was again found that the volume of erythrocytes is the same, or very nearly the same, in all solutions isosmotic with the natural medium of the erythrocyte. Hedin's (42) determinations of the relative osmotic pressure of solutions of various crystalloids, i.e., his determinations of the van't Hoff factor i, are in fair agreement with values obtained by Koeppe using a similar method, with those of Hamburger obtained by the hemolysis method discussed in the preceding paragraph, and with those of deVries using plant cells as osmometers. Moreover these values agree with physico-chemical measurements such as freezing point determinations by Raoult and by Arrhenius, and with the electrical conductivity measurements of Kohlrausch, van't-Hoff and Reicher, and Gregory. Therefore in these experiments the exchange of water between the erythrocyte and its surrounding medium is governed by the Avogadro-van't Hoff law.

c. The experiments just discussed suggested the question: Is the volume of erythrocytes the same in all solutions isosmotic with each other, but of different osmotic pressure from the blood plasma? The following table gives the results of experiments on volumes of erythrocytes obtained by Hedin (41a) with the hematocrit in isosmotic solutions of KNO_3 and $NaNO_3$ over a considerable range of concentration.

CONCENTRATION	KNO_3	$NaNO_3$
mols per liter		
0.1	39.2	39.3
0.13	36.2	36.2
0.15	35.2	35.2
0.17	35.1	35.2
0.2	34.4	34.5
0.3	31.4	31.4
0.4	29.6	29.6

As the table shows, the volume is the same in isosmotic solutions of KNO_3 and $NaNO_3$ in several concentrations. However, when two salts with different anions were compared, the agreement was not complete; the volume of erythrocytes in hypotonic NaCl solution was invariably slightly greater than in hypotonic KNO_3 solutions of like osmotic pressure; likewise, in hypertonic solutions of these two salts the volume of the corpuscles was greater in NaCl solutions.[4] These ex-

[4] On the contrary, Koeppe (62) stated that the volume of erythrocytes is identical in all isosmotic solutions, no matter whether they be isosmotic with the blood, or of greater or lesser concentration.

perimental findings of Hedin and similar results of others apparently showed that the volume of red blood cells under certain conditions is not determined only by the osmotic pressure of the solution. One possible source of error in the hematocrit determination of Hedin, Koeppe and many others, is that centrifugation was not sufficiently prolonged.

Another reason for such discrepancies was recently pointed out by Ege (15). Using a much improved hematocrit method and carefully determining the freezing point of his solutions he again investigated the volume of red cells in isosmotic solutions of various concentrations. The mean values of six determinations are given in the following table:

DEPRESSION OF FREEZING POINT OF SOLUTION	VOLUME IN KNO_3	VOLUME IN NaCl	DIFFERENCE
			per cent
0.579	98.1	103.8	5.8
0.460	114.0	120	5.3
0.368	134.0	137.2	2.4

The results of Ege also indicate a small but definite difference in volume of the red cells in isosmotic solutions of KNO_3 and NaCl.

Now the law of Avogadro-van't Hoff can be applied only to solutions of substances that do not penetrate into the cell. Ege (15), in carefully controlled experiments, showed that apparent deviations in the osmotic behavior of erythrocytes can adequately be accounted for by the fact that this cell is permeable to a variety of anions, and that the rates of penetration (or exchange) of anions differ. For example Cl^- penetrates (or is exchanged) somewhat more rapidly than NO_3^-. When, therefore, erythrocytes are placed in isosmotic solutions of these two salts, the cells in the chloride solution will, at first, swell to a greater extent because of the more rapid penetration (or exchange) of the Cl ion. The volume of erythrocytes in isosmotic solutions of NaCl and KNO_3 will be the same only when anion equilibrium has been established.

This fact may be illustrated by the following experiment. Erythrocytes were placed in 1/8 molar solutions of KNO_3 ($\Delta = 0.450$) and of NaCl ($\Delta = 0.452$), and volumes were determined at different times after immersion. In the following table volumes of erythrocytes are expressed in relative values (the volume of the first determination in KNO_3 solution being taken as 100).

KNO_3		NaCl	
minutes		*minutes*	
7	100	5	106.0
30	104.3	56	109.6
224	109.6	120	108.9
360	109.1	320	108.8

It is seen that the volume of the cells increases more rapidly in the chloride solution than in the isosmotic solution of the nitrate, but that in both approximately the same volume is finally attained. In solutions of non-electrolytes, in which no anion exchange complicates the situation, the volume of the cells does not vary at different times after immersion. Moreover if by repeated washing in suitable isotonic solutions, anion equilibrium between cell and surrounding medium is first established, no difference in volume is obtained in isosmotic solutions of NaCl and KNO_3. The apparent deviation in the osmotic behavior of erythrocytes from the Avogadro-van't Hoff law, occurring in special cases, is therefore easily accounted for.

Experiments with muscle. a. As in the case of plant cells it is easily possible (by placing comparable muscles in a series of solutions of different concentrations) to determine the exact strength of a solution which is isotonic with the muscle, that is to say, a solution in which the muscle neither loses nor gains water. Thus resting gastrocnemius muscle of the frog generally undergoes no significant changes in weight, during the first hour, in a solution of NaCl containing 0.7 gram in 100 grams of water, or, approximately, an M/8 solution (Loeb, 75; Overton, 99[5]).[6] In solutions of NaCl of less than 0.125 molar concentration muscle gains water, (and therefore weight), while in a more concentrated solution water is lost. That the exchange of water between muscle and surrounding solutions follows Avogadro's law is shown in the following experiment. A 0.125 M. NaCl solution has the same osmotic pressure as a 0.231 M. solution of a non-electrolyte. Loeb (80) placed muscles

[5] Hill (44) recently determined by several methods the osmotic pressure of frog's muscle as equivalent to an 0.725 per cent NaCl solution.

[6] The concentration of NaCl solution isotonic with muscle varies considerably (between 0.6 to 1.0 per cent or more) depending on the previous history of the frog's muscle. For example, previous activity causes a rise of the osmotic pressure of muscle owing to the production of new molecules in solution, such as lactic acid. (For a general discussion see A. V. Hill (44), Loeb (75) and Fletcher (29).) It is to be emphasized that in order to have comparable material frogs should be used which have been kept for at least 24 hours under as nearly as possible the same conditions of activity, light, water, temperature, oxygen supply, etc. (Loeb, 75).

in a series of solutions of three sugars of very different molecular weight, —a mono-saccharide, grape-sugar; a disaccharide, cane-sugar; and a tri-saccharide, raffinose. Changes in weight of the muscle were determined after 1 hour immersion. It was found, for all three sugars, that in 0.2 M. solution the muscles gained weight, while in 0.25 molar (and in still greater concentrations) they lost water. As Loeb points out, the turning point between loss and gain in weight lies between the limits to be expected if osmotic forces govern the exchange of water.

In other experiments the same investigator found (75) that the weight of muscle is the same in M/8 LiCl or KCl solution as in a NaCl solution of the same concentration. The degree of dissociation in these three salts is about the same. Still more decisive proof of the applicability of the Avogadro-van't Hoff law to muscle was brought by Loeb when he showed that solutions of LiCl, KCl, RbCl, CsCl, $MgCl_2$, $CaCl_2$, $SrCl_2$, and $BaCl_2$ bring about approximately the same change in weight as a NaCl solution of the same osmotic pressure, provided that the duration of the experiment is short. However, after injury or death of muscle cells the different solutions bring about very different changes in weight.

b. That Avogadro's law governs the osmotic behavior of muscle not only in isosmotic solutions which are at the same time isotonic with the cell contents, but also in isosmotic solutions which are not isotonic with the cell is shown by experiments of Webster (126), and especially of v. Körösy (64). The latter determined the weight change of frog's muscle in a series of isosmotic solutions (calculated from conductivity data) of dextrose, sucrose, NaCl, KCl, and $CaCl_2$, over a wide range of concentrations, as shown in the accompanying table. The figures express per cent change in weight after 1 hour's exposure.

TOTAL EFFECTIVE OSMOTIC CONCENTRATION	DEXTROSE	SUCROSE	NaCl	KCl	$CaCl_2$
0.05	+30	+35	+37	+32	+22
0.15	+9	+13	+11	+12	+9
0.20	+2	+6	+4	+6	+3
0.22	0	+2	+2	+3	0
0.23	+1	+2	+1	+3	−2
0.24	0	+2	0	+4	−3
0.26	+1	0	0	+3	−2
0.31	−2	−2	−2	0	−5
0.41	−6	−7	−6	−3	−5
0.61	−9	−12	−9	−6	−10
1.0	−14	−21	−11	−8	−9
2.0	−27	−34	−16	−11	−12

Considering the variability of the material, and the fact that the calculations probably introduce some error, the agreement at least in the lower concentrations is good. The table shows that the Avogadro-van't Hoff law holds for muscle not only near the point of isotonicity but over a considerable range of concentration. After exposures of longer than 1 hour considerable deviations were observed, and after 17 hours, i.e., after injury or death of the material, there was no longer any agreement.

Similar results were obtained by Webster (126). Thus the weight changes after 1 hour's immersion in M/8, M/16, and M/32 solutions of NaCl, KCl, and NH_4Cl were practically the same and agreed with the changes observed in solutions of sucrose having the same osmotic pressure as the above. This is shown in the accompanying table.

CONCENTRATION OF		NaCl	KCl	NH_4Cl	SUCROSE
Salts	Sucrose				
M/8	0.23 M.	0	+4	+1	+1
M/16	0.12 M.	+13	+20	+14	+11
M/32	0.05 M.	+25	+25	+27	+26

After more than 1 hour's immersion the weight changes in the solutions of the different substances showed much variation.

Summary: The experiments reviewed in the present section make it apparent that both plant and animal cells obey the law of Avogadro-van't Hoff as long as they remain alive and uninjured, i.e., as long as the semipermeability of their surfaces is preserved. With living material which permits accurate measurement of volume it has been shown that *a*, the cell may be employed as an osmometer for the determination of molecular weight of a non-penetrating, non-injurious substance; *b*, the cell may be used to determine the relative osmotic pressure of solutions of various non-penetrating, non-injurious electrolytes and non-electrolytes, and the results of such determinations are in good agreement with those obtained by physico-chemical methods; *c*, the cell has the same volume (or weight) in all isosmotic solutions of non-penetrating, non-injurious substances, no matter whether such isosmotic solutions are of the same osmotic pressure as the natural medium of the cell, or of different pressure. Such solutions have no other quality in common than the same number of molecules or ions.

B. *Applicability of the law of Boyle-van't Hoff to cells.* The law of

Boyle-van't Hoff states that the osmotic pressure of a solution varies inversely with the volume of solvent in which a definite quantity of the solute is dissolved; in other words, the product of pressure and volume is constant, $PV = K$. Alternately stated, the osmotic pressure is proportional to the concentration (number of particles per unit volume) of a solution, at constant temperature, $P = C \times K'$.

This relation is strictly true only of ideal solutions. Application of the law to living cells will enable us to determine how closely the contents of cells approximate in their properties to ideal solutions. If the contents of the cells are sufficiently dilute to permit application of this law, then the total volume of the cell should vary inversely with the osmotic pressure of the external solution with which it is in osmotic equilibrium.

Though the juice in sap vacuoles of certain plant cells might be expected to have properties closely resembling those of dilute electrolytes, such a state cannot be anticipated in the case of other cells, indeed, of any animal cells. The latter all contain varying amounts of such substances as proteins and fats, which obviously can exert comparatively little or no osmotic pressure. The volume occupied by such osmotically "inactive" substances must therefore be subtracted from the total cell volume before computing the pressure volume product.[7] Failure to do so has led some observers to deny unjustifiably that the law of Boyle-van't Hoff is obeyed by cells.

Another consideration of prime importance is that the semipermeability of the cell must be intact. Obviously a cell swelling in a hypotonic solution or shrinking in a hypertonic one will not attain the volume at equilibrium predicted by the law of Boyle-van't Hoff if dissolved substances are able to escape from the cell in the one case or enter it in the other. Semipermeability is maintained only as long as the cell is alive and uninjured. Failure to maintain the cell in normal condition has been another fruitful source of error and has led some investigators to conclude that the law of Boyle-van't Hoff is not obeyed.

Thus deviations from the Boyle-van't Hoff law reported by M. H. Fischer appear to depend on the factors discussed above.[8]

[7] Under the term osmotically "inactive" material is also included "bound" water if any exist. In the more exact theory the volume occupied by the solutes themselves should also be subtracted.

[8] For a recent summary of Fischer's views in which his various papers are cited see (26); for criticism see especially Beutner (6, 7), Winterstein (127), Ege (17, 20) and Höber (47).

1. Experiments with erythrocytes and other isolated cells. Probably the most extensive, and until very recent times most complete studies are those of Hamburger (38) and of Koeppe (62, 63). These experiments paved the way for a proper understanding of the osmotic behavior of cells. Hamburger, using an hematocrit method for determining volume change, found the following relative volumes of erythrocytes in sodium chloride solution.

CONCENTRATION	RELATIVE VOLUME OF ERYTHROCYTES	PER CENT CHANGE
0.7 per cent NaCl	43.5	+13.0
Serum (isotonic with 0.9 per cent NaCl)	38.5	
1.0 per cent NaCl	36.75	−4.54
1.5 per cent NaCl	31.75	−17.5

Hamburger reasoned that if the erythrocytes consisted solely of a salt solution surrounded by a semipermeable membrane, and if such cells obeyed the law of Boyle-van't Hoff, greater volume changes would be expected than those actually obtained. For example, on transferring a cell from 0.9 per cent NaCl solution to 0.7 per cent NaCl solution the swelling should be $\frac{0.9 - 0.7}{0.9} \times 100 = 22$ per cent. The data given show that volume changes in either swelling or shrinking fall considerably below the expected value. Similar results were obtained with leucocytes and spermatozoa. From experiments of this type Hamburger reached the conclusion that the erythrocytes (and other cells) contain a substance or substances which take no part or but a very slight part in osmotic exchange of water. He pictured the cell as consisting of a firm osmotically inactive "Gerüst" between which lies the intracellular fluid, and he clearly pointed out that it is only the intracellular fluid which is concerned in osmotic volume change of cells.[9]

We shall consider the calculation of the osmotically "inactive" material in connection with later work.

2. *Experiments with muscle and kidney.* If muscles, such as the gas-

[9] Hamburger's (38, 40) term "Gerüst" means in the case of the erythrocyte chiefly hemoglobin. Various writers have applied the terms "dispersed phase" (Ege (17, 21)), (Wieringa (130)); "non-solvent space," (Netter (93)); "non-liquid phase" (Jacobs (58)); "osmotically inactive substance" (McCutcheon, Lucké and Hartline (90)); "dry substance" (Overton (99)), etc., to that fraction of the cell volume which does not take part in osmotic exchange of water.

trocnemius or sartorius of frogs, are placed in a graded series of hypotonic solutions, the tissue does not take up water (as determined by weighing) in proportion to the difference in osmotic pressure between the cell and the muscle. Similarly in hypertonic solution muscle does not shrink in proportion to the difference in osmotic pressure. These apparent deviations from Boyle's law have been observed by many investigators, and the experiment of v. Körösy (64) and of Webster (126), already cited, may be referred to as representative of results reported by Loeb (75), Overton (99), Cooke (9), Siebeck (110), Fletcher (29), Beutner (6, 7) and others. With tissues other than muscle similar deviations from theoretical volume changes are obtained. Thus Siebeck (110), using the small kidneys of frogs in Ringer's solutions of graded anisotonicity, obtained the following results:

RELATIVE OSMOTIC PRESSURE	RELATIVE MEAN WEIGHT OF KIDNEYS	PRODUCT
2.00	79	158
1.00	100	100
0.75	111	83
0.50	127	64
0.25	174	44

These figures show that while the weight of the kidney varies with change in the concentration of the medium it is not inversely proportional to the osmotic pressure.

It is obvious, then, that an entire muscle or an entire kidney does not behave as if it were composed of numbers of miniature osmometers, free to undergo volume changes. One of the principal explanations for the apparent deviations from Boyle's law in the case of tissues is that offered by Overton (99) who held that a fraction of the total amount of water within the cells exists in "bound" form, and is therefore incapable of being shifted under the driving force of osmotic pressure. The question of the state of water in tissues has of late years received considerable attention. (For fuller discussions see A. V. Hill (44, 45), Gortner (32, 33)). Cramer (10) in a recent discussion before the Faraday Society formulates the problem as follows, "Is the water content of a living cell dependent entirely on osmotic forces or is it dependent also on the inhibition of the lyophilic colloids of the cell content?" In the former case the water would be entirely "free", in the latter, a fraction of the water would be "bound" and incapable of taking part in osmotic ex-

change. However, as Gortner (33) points out, it is probable that there is "an insensible gradation from water which is an integral part of protoplasmic structure to that which is simply the medium in which the protoplasm is suspended." Unfortunately there is no unanimous usage of the terms "free" and "bound" water, and these terms, at present, must be defined arbitrarily. Various methods have been employed to measure the "free" or "bound" water of biological fluids or of cells and tissues. The two principal methods are those used by Gortner and his associates and by A. V. Hill and his associates.

In the case of fluids (blood) or blood cells, Hill (44) dissolved a known quantity of some suitable substance in a known amount of fluid and measured the depression of vapor pressure by a very delicate thermal method; this value was then compared with the value obtained by adding the same substance to approximately isotonic salt solution. With tissue (muscle) a weighed quantity was stirred with a known amount of a hypertonic salt solution, and the change of vapor pressure of the fluid measured.

Gortner (33), using the juice expressed from certain plant tissues, determined the freezing point. In a quantity of the juice containing exactly 10 grams of total water was dissolved 0.01 M. of sucrose and a second determination of the freezing point was made. If all of the 10 grams of water in the sample were free to dissolve the sucrose, the solution would be exactly molar in concentration; the second determination of the freezing point should give a difference from the first equal to the value characteristic of a one molar sucrose solution. If, however, a fraction of the 10 grams of total water were "bound," less water would be free to dissolve the sucrose and the depression of the freezing point would be in excess of that corresponding to a molar solution. The methods of A. V. Hill and of Gortner should lead to similar results. Gortner finds, however, that in certain plant tissues a considerable fraction of the total water is "bound," while Hill finds very little if any water of blood or muscle is in a "bound" state. Further studies are therefore necessary in order to decide whether in a given cell or tissue all of the water is free to move under the influence of osmotic forces or whether some fraction is incapable of doing so. In any case, according to Hill, in muscle the free water corresponds very nearly to total water fraction. Hill (44) accounts for the failure of muscle to reach the theoretically expected volume in an isotonic solution by *a*, the loss of semi-permeability in a considerable fraction of the total number of component cells as the result of removal from normal environment; *b*,

progressive swelling or shrinkage of the tissue owing to colloidal changes in the dead fibers; *c*, slowness of attaining equilibrium.

In addition it is likely that part of the discrepancy may be accounted for by the presence of osmotically "inactive" material other than "bound" water.

We shall now consider the evidence to the effect that with suitable material and under proper experimental conditions volume changes of the cells follow the course predicted by Boyle's law.

3. *Experiments with plant cells.* With certain cells having regular geometrical form (for example, the parenchymatous cells of the stem of *Tradescantia elongata*), volume changes of the plasmolyzed protoplast may be accurately measured by a simple method due to Höfler (48, 49). Höfler showed that at equilibrium the volume of the plasmolyzed protoplasmic sac (which may now be likened to a "naked" cell) is exactly inversely proportional with the concentration of the medium which has caused plasmolysis.

If, therefore, in a hypertonic solution of known concentration C_1 the protoplasmic sac has the volume V_1, it will assume the volume V_2 when the concentration is changed to C_2, so that $C_1/C_2 = V_2/V_1$ or $C_1 \cdot V_1 = C_2 \cdot V_2 =$ constant. Upon these principles is based Höfler's plasmometric method for determining the osmotic pressure (osmotic value) of plant cells. If, for example, in a given hypertonic solution of *known* concentration the protoplasmic sac shrinks to 3/4 of its original volume, the osmotic pressure of its contents must rise to 4/3 of the value before plasmolysis occurred. The osmotic pressure of the non-plasmolyzed cell is therefore 3/4 that of the plasmolyzing solution.

The accuracy with which the volume pressure relation obeys the Boyle-van't Hoff law is shown in the experiment cited below. The figures in the first column represent four gram-molecular solutions of sucrose of different concentration; the figures in the second column, the mean relative volumes (ratio of volume of plasmolyzed and non-plasmolyzed protoplast) of eleven neighboring cells of *Tradescantia*, while the last column shows the constancy of the product, concentration and relative cell volume.

CONCENTRATION OF SUCROSE MOLS C	RELATIVE VOLUME OF PLASMOLYZED CELL V	ISOTONIC CONCENTRATION CALCULATED FROM $C \cdot V$
0.30	0.585	0.175
0.35	0.494	0.173
0.45	0.382	0.172
0.60	0.287	0.172

The figures are significant to the second decimal. The constancy of the product C·V shows that the cells under the condition of the experiment possess true semipermeability, attain true equilibrium, exhibit no evidence of injury, and that the Boyle-van't Hoff law is fully obeyed in the concentrations employed.

Höfler (48, 49) points out that for cells with abundant protoplasm a correction must be applied; see also the paper by Walters (125).

Höfler's plasmometric method permits very exact determination of the osmotic pressure of plant cells. For details of technique his original papers should be consulted, as well as the articles by Grafe (35) in Abderhalden's Arbeitsmethoden.

4. *Experiments with egg cells of Echinoderms and other marine invertebrates.* One of the best osmometers that has yet been found is the egg of certain sea urchins. The eggs of several species are spherical. Such a definite geometrical shape is a great advantage, for the volume of the cell can be found simply by measuring the diameter by means of microscope and ocular micrometer. Since change in volume in anisotonic solutions (of non-penetrating substances) is equal to the volume of water that has entered or left the cell, we thus have a direct and accurate method of measuring exchange of water between cell and medium.

Sea urchin eggs offer several other advantages. A great number of cells varying but little in osmotic properties can be obtained from a single animal. Their small size—about 74 micra in diameter in the case of *Arbacia punctulata*—allows rapid equalization of osmotic pressure within the cell. The spherical shape is maintained for hours in hypotonic solutions; it has been shown for *Arbacia* that no flattening of the cell occurs either from its own weight or other cause (90).

A prerequisite for an ideal osmometer is that the membrane should be perfectly semipermeable, i.e., should allow passage of solvent but not of solute. If this condition is not realized it is evident that the amount of swelling of the cell in hypotonic solutions and of shrinking in hypertonic solutions would be less than expected, i.e., the law of Boyle-van't Hoff would not hold. There is evidence in the case of *Arbacia* that, under suitable experimental conditions, semipermeability is practically perfect. This evidence is that when cells are swollen in hypotonic sea water and then shrunken in ordinary sea water, they return to their original volume (90). This could be the case only if there occurred no leakage of salts or if loss of salts during swelling were just balanced by gain of salts during shrinkage. Such an exchange is of course possible, but, as will be seen below, it must be slight if it occurs at all.

However, this high degree of semipermeability is preserved only as long as the cell is uninjured. As will be shown later, injured cells lose their semipermeability to a greater or less extent and behave in hypotonic solutions quite differently from normal cells. One of the greatest advantages of this material (sea urchin eggs) is that with them injury may be accurately defined and determined. At the end of an experiment, cells are returned to ordinary sea water and inseminated. Cells are regarded as normal if they undergo typical cleavage; injured, if cleavage is atypical or absent (85).

We are now ready to consider whether sea urchin eggs obey the law of Boyle-van't Hoff.

a. Earlier observations of Lillie (70) and of McCutcheon and Lucké (87) indicated that unfertilized eggs of *Arbacia* fall short of attaining predicted volumes when in equilibrium with hypotonic solutions.

b. The subject was investigated more fully by Fauré-Fremiet (24) with the eggs of *Sabellaria alveolata*. These were placed in solutions of saccharose of known osmotic pressure and cell volume determined after equilibrium had been attained.

Results of such an experiment are given in the accompanying table. When pressure (column 1) is multiplied by volume (column 2), the product (column 3) shows a decided drift.

1	2	3	4	5
PRESSURE	VOLUME OF EGG	PV	VOLUME OF H_2O	$P \cdot V$ OF H_2O
Δt	$cm.^3$		$cm.^3$	
0.566	34.4×10^{-8}	19.5	29.85×10^{-8}	16.9
1.135	21.9	24.9	17.35	19.7
1.760	15.25	26.8	10.70	18.8
2.598	11.87	30.8	7.32	19.0
3.730	9.95	37.1	5.40	20.1
5.100	8.29	42.3	3.74	19.1

Fauré-Fremiet considered that this result was due to the presence in the cell of material exerting little or no osmotic pressure. What would be the result if only the volume of osmotically active solution were considered? This volume would not be greatly different from the volume of water in the cell. The latter quantity was determined by subtracting volume of dry substance from total cell volume. He found that the cell contains 2.256 grams of water for every gram of dry substance. Assuming the specific gravity of dry substance as approximately equal

to 1, 30 per cent of cell volume consists of dry substance. In isotonic solutions the cell volume is 15.15×10^{-8} cm.^{3} of which 4.55×10^{-8} cm.^{3} is dry substance.

When this latter volume is subtracted from observed volumes of column 2, volumes of water are found (column 4). On multiplying the latter by pressures (column 1) the products of column 5 are obtained. It is seen that now the product is practically constant, except in the case of the most hypotonic solution. (Columns 3, 4 and 5 have been computed by the present writers.)

Thus *Sabellaria alveolata* is seen to obey the Boyle-van't Hoff law. Objection may be raised that the percentage of dry substance cannot be determined accurately with such material, but this objection is met by recalculating Fauré-Fremiet's data without considering dry substance. Before doing this, other work is to be reviewed.

c. The osmotic properties of *Arbacia punctulata* were investigated by McCutcheon, Lucké and Hartline (90). The applicability of the Boyle-van't Hoff law to this material was tested in order to lay the basis for a kinetic theory of osmosis, which will be developed in the next chapter of this review. It is therefore necessary at this point to discuss the application of the law of Boyle-van't Hoff to such a system as the sea urchin egg.

In the theory of the ideal, dilute solution, the osmotic pressure of a given amount of dissolved substance is inversely proportional to the volume in which it is distributed. The assumption that the solutions of the present system behave as ideal, dilute ones is an approximation which permits the application of the simple laws governing osmotic processes. An attempt to evaluate the activities of all the constituents of the cell and its medium is of course out of the question.

We may, therefore, write

$$PV = K \qquad \text{(I)}$$

where P is the osmotic pressure of the dissolved substances in the egg, V is the volume of the egg, and K is a constant. From the consideration that the egg initially must be in equilibrium with ordinary sea water, of known osmotic pressure, it is seen that K may be determined experimentally by measuring the volume of the egg as it rests in its normal medium. We write for (I) its equivalent:

$$PV = P_o V_o \qquad \text{(I a)}$$

where P_o is the osmotic pressure of ordinary sea water and V_o the volume of the egg in its normal medium.

This equation furnishes a method for determining, at any time, the osmotic pressure in the interior of the egg. In addition, it furnishes the law that the equilibrium volumes of the egg must follow in anisotonic solutions. In these cases the internal pressure must equal that of the external medium, and the expected volume of the egg at equilibrium is given by

$$P_{ex} V_e = P_o V_o, \qquad \text{(I b)}$$

where P_{ex} is the osmotic pressure of the external medium (known from its concentration) and V_e is the expected equilibrium volume of the egg. (I b) therefore furnishes the means of determining whether the Boyle-van't Hoff law is obeyed by this osmometer.

The solutions employed in these experiments were undiluted sea water (100 per cent), 90 per cent (i.e., 90 parts of sea water with 10 parts of distilled water) 80, 70, 60 and 50 per cent. Fifty cells in equilibrium with each of these solutions were measured by each of two observers.

After measurement, cells (in two of the experiments) were returned to ordinary sea water and sperm was added. Normal cleavage took place, except in the case of cells taken from 50 per cent sea water; these showed atypical cleavage.

The combined results of three experiments are recorded in the appended table.

(1)	(2)	(3)	(4)	(5)	(6)
RELATIVE PRESSURE	VOLUME OBSERVED	PV	$P(V-b)$	VOLUME CALCULATED FROM $V_e = \frac{V_o P_o}{P_{ex}}$	VOLUME CALCULATED FROM $V_e = \frac{(V_o-b)P_o}{P_{ex}} + b$
1.0	2,121	2,121	1,881	2,121	2,121
0.9	2,316	2,084	1,868	2,357	2,328
0.8	2,570	2,056	1,864	2,651	2,590
0.7	2,922	2,045	1,878	3,019	2,925
0.6	3,420	2,053	1,909	3,535	3,374
0.5	4,002	2,002	1,881	4,241	3,999

In the first column are given relative pressures, in the second observed volumes and in the fifth column, volumes calculated by equation (I b). The combined data are presented graphically in figure 1, where the lower curve represents observed volumes of 300 cells plotted against the relative pressure (or concentration) of the outside solution, and the

upper curve is calculated by equation (I b). It is seen that there is a slight deviation of the two curves which becomes more marked as we pass from higher to lower pressures. But even in 50 per cent solution the cells fail by less than 6 per cent to attain the volume calculated. Therefore equation (I b) proves to be a rather fair first approximation.

The discrepancy, however, is a real one. In column 3 of the table are given the pressure volume products of each experiment. In each case it is seen that there is a slight but definite drift in values of PV.

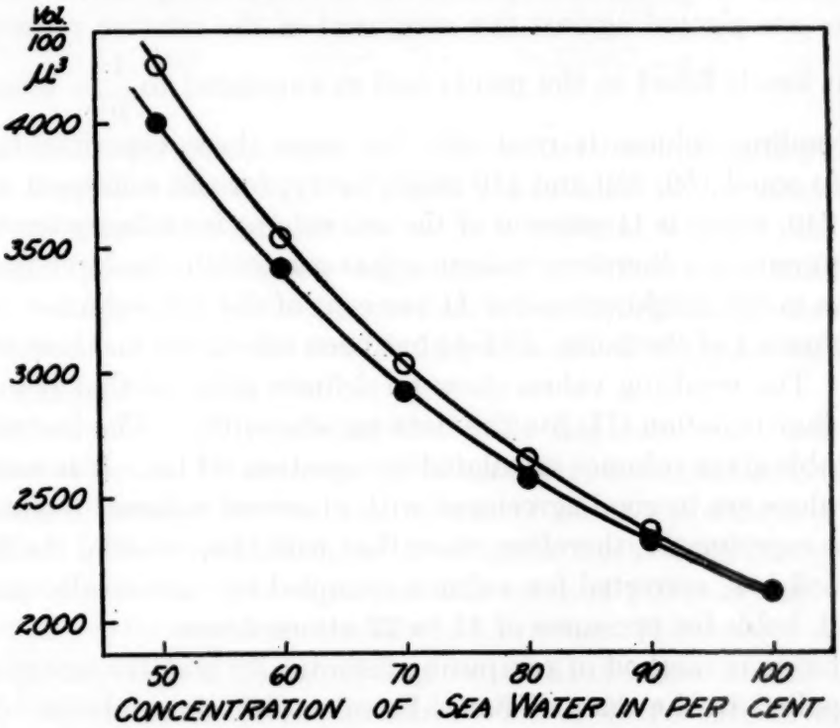

Fig. 1. Comparison of observed and calculated volumes. Solid circles represent mean volumes of 300 cells in osmotic equilibrium with various dilutions of sea water. Open circles are volumes calculated by equation (I b).

There are a number of factors which might prevent the cell from swelling as much as predicted. Of these the one that is surely important and which might account for practically the entire discrepancy is that obviously a considerable fraction of the cell volume is occupied by material that can exert little or no osmotic pressure. This fact is best demonstrated by rapidly centrifuging the eggs, whereupon zones of oil and pigment granules separate from the rest of the cytoplasm. The osmotically active solution therefore occupies a volume which is equal not to V but to V-b, in which b is the volume occupied by osmotically inactive material.

Equations (I) to (I b) now become, respectively,

$$P(V\text{-}b) = K \qquad \text{(II)}$$
$$P(V\text{-}b) = P_o\,(V_o\text{-}b) \qquad \text{(II a)}$$
$$P_{ex}\,(V_o\text{-}b) = P_o(V_o\text{-}b) \qquad \text{(II b)}$$

The value of b may be found by substituting in equation (II b) the values given in columns 1 and 2 of the table. Since this method of computing gives a considerable scattering of values for b, a graphic method is preferred, one in which all points can be weighed equally. Observed volumes are plotted against the reciprocal of the relative pressures, a straight line is fitted to the points and extrapolated to $\frac{1}{p} = o$, and the corresponding volume is read off. In these three experiments, b is found to equal 150, 250 and 310 respectively; for the combined data, b equals 240, which is 11 per cent of the cell volume in ordinary sea water. This computation therefore indicates that osmotically inactive material occupies in the neighborhood of 11 per cent of the cell volume.

In column 4 of the table, $P(V\text{-}b)$ has been calculated for these experiments. The resulting values show no definite drift, so that it may be stated that equation (II) fits the data satisfactorily. The last column of the table gives volumes calculated by equation (II b). It is seen that these values are in good agreement with observed volumes (column 2).

These experiments, therefore, show that with this material the Boyle-van't Hoff law, corrected for volume occupied by osmotically inactive material, holds for pressures of 11 to 22 atmospheres.

d. The same method of computing osmotically inactive material has been applied to the data of Fauré-Fremiet (24) given above. When percentage volume change is plotted against the reciprocal of relative pressure, a linear relation is found, indicating that the pressure volume product is constant when allowance has been made for osmotically inactive material. The percentage of the latter is given by the graph as 31, which is in remarkably good agreement with 30 per cent of dry weight reported by Fauré-Fremiet.

e. In the case of unfertilized eggs of the sea urchin, *Paracentrotus lividus*, Ephrussi and Neukomm (23) reported that the law of Boyle-van't Hoff is not obeyed. This conclusion was reached by multiplying the pressure of the solution by the volume of water in the egg. The latter figure was found by subtracting the dry weight of the egg from the total weight. Unfortunately, the dry weight is not stated.

However, the data in the accompanying table, in which relative vol-

ume of the entire cell instead of volume of water is given, permit us to plot cell volume against the reciprocal of osmotic pressure. The resulting graph is linear, in fact the fit is excellent.

PRESSURE ATMOSPHERES	PER CENT VARIATION IN VOLUME OF EGGS
9	+98.3
17	+28.1
23	+5.2
25	0
27	−3.5
29	−6.5

Taken at face value the graph indicates that the law of Boyle-van't Hoff is obeyed. However, the graph states that 46 per cent of cell volume is occupied by osmotically inactive material, a figure that seems unreasonably high. Similarly high figures have been obtained by the present writers with *Arbacia punctulata* when injury has occurred. It remains a matter of doubt how the experiments of Ephrussi and Neukomm should be interpreted.

5. *Experiments with erythrocytes.* While the volume of individual erythrocytes cannot be measured with accuracy, the hematocrit method permits measurement of the total volume of all of the cells in a given suspension. We have already referred to the work of Hamburger (38) and others (41, 63) who employed this method for studying volume changes of erythrocytes in anisotonic solutions. Hamburger was the first to point out that the law of Boyle-van't Hoff is not directly applicable to the osmotic volume—pressure relation in the case of erythrocytes, but that allowance must be made for that part of the volume of these cells occupied by non-liquid phase (i.e., mainly hemoglobin).

It is necessary therefore to determine accurately the absolute value of the fraction of the cell volume occupied by hemoglobin and other material that can exert little or no osmotic pressure. This was done by Ege (17, 21). He found by direct determination of dry matter a value for the "dispersed phase", b, of about 35 volume per cent of the cell (in non-injurious, non-penetrating isotonic solution). Ege further showed that little, if any, water is "bound" to the protein of the erythrocyte. He next compared the value of b thus obtained with the value calculated from the equation,

$$P_o(V_o - b) = P_1(V_1 - b)$$

where V_o is the volume at equilibrium of the erythrocytes in an isotonic medium of osmotic pressure, P_o, and V_1 is the volume at equilibrium in a

hypotonic or hypertonic medium having the osmotic pressure P_1. As has already been pointed out the value of b may be obtained arithmetically by substitution in the above equations, giving $b = \frac{P_1V_1 - P_oV_o}{P_1 - P_o}$; or graphically by plotting volumes against the reciprocals of osmotic pressure as discussed above. Ege found the calculated value of b to be slightly greater than the value obtained by direct determination. By assuming the value to lie between 35 and 40 volumes per cent of the erythrocyte (in an isotonic medium) the observed volume changes in anisotonic solutions closely approached the volumes calculated according to the law of Boyle-van't Hoff. This relation may be illustrated by the following experiment.

Rabbit's erythrocytes were brought into equilibrium with sucrose solutions of graded concentration, and centrifuged until no further volume changes were observed. In the table given below the concentrations are expressed as relative pressures, 1.5 meaning a solution of 150 grams of sucrose plus 1 liter of distilled water. The volumes observed are expressed as relative volumes.

(1)	(2)	(3)	(4)	(5)	(6)	(7)
RELATIVE PRESSURE (P)	VOLUME OBSERVED	RELATIVE VOLUME OBSERVED (V)	$P \cdot V$	$P(V-b)$	VOLUME CALCULATED FROM $V_c = \frac{P_oV_o}{P_{ex}}$	VOLUME CALCULATED FROM $V_c = \frac{P_o(V_o-b)}{P_{ex}} + b$
1.5	1,163	78.3	174.5	57.5	66.7	80.0
1.0	1,477	100.0	100.0	60.0	100.0	100.0
0.75	1,744	118.2	88.5	58.7	133.4	120.0
0.64	1,906	129.0	82.5	57.0	155.6	133.3
0.562	2,135	144.3	81.1	58.6	178.0	146.7
0.50	2,207	153.0	76.5	56.5	200.0	160.0

It is seen that the product of P and V shows a very considerable drift, while the product of $P(V\text{-}b)$ is nearly constant. The last column of the table shows the excellent agreement between observed and calculated volumes at equilibrium when the value of b = 40 per cent of V_o is introduced in the equation. By contrast, the equilibrium calculated without taking into account the fraction of the volume occupied by osmotically inactive material shows great deviation from the observed values. Ege's value of the dispersed phase, b, of the blood corpuscle is lower than that given by Hamburger (38), who calculated that about 50 to 55 per

cent of the total cell volume is occupied by osmotically inactive material. Still higher figures were obtained by Gough (34) who estimated that the watery phase in which the crystalloids are dissolved forms only one-third of the volume of the erythrocyte. Ege (21) in discussing the findings of Hamburger and Gough points out that incomplete sedimentation of the erythrocytes during the process of centrifugation introduces a systematic error of considerable magnitude. He shows by calculation that the method of determining the dispersed phase employed by Hamburger and Gough will thus easily lead to excessively high values unless the centrifuging is continued for a sufficient length of time so to pack the corpuscles as to leave no interstitial fluid between them. He again states that by means of various methods he has consistently found the volume of the non-liquid phase to approach nearly the percentage of dry matter of the corpuscles.

In this connection reference should be made to the recent work of Krevisky (65) whose figures for b support those of Gough, namely, that only 30 to 35 per cent of the volume is occupied by free or osmotically transferable water, while 30 to 35 per cent of the water is bound to the proteins and hence incapable of taking part in the water exchange between cell and surrounding medium. The question of the state of water in the erythrocyte is so thoroughly discussed by A. V. Hill that the reader is referred to his papers (44, 45). Hill by using very exact methods of vapor pressure measurements concluded that the "free water of the blood or of centrifuged corpuscles is practically equal to the total water." It may be pointed out that in our opinion the high values of Krevisky are possibly due to loss of semipermeability of the erythrocytes, which in Krevisky's experiments were centrifuged in strongly anisotonic NaCl solutions (3 to 20 per cent NaCl).

Because of the discrepancy between the value of b obtained on the one side by Ege and on the other by Hamburger, Gough and Krevisky, the question of the quantity of free water in the erythrocyte was lately reinvestigated by Schiodt (107) who also used a hematocrit method. His determinations confirm those of Ege. In hypotonic, isotonic and slightly hypertonic solutions of NaCl Schiodt calculated from the volume-concentration curves the quantity of free water to be about 65 per cent; or, in other words, the value of b was found to be about the same as reported by Ege (35 to 40 per cent). In very hypertonic solutions only were higher values obtained. We are therefore inclined to regard Ege's original determinations as correct.

6. *Experiments with yeast cells.* Wieringa (130) investigated the

applicability of the Boyle-van't Hoff law to osmotic volume changes in yeast cells. Employing a centrifuge method, for the details of which his original paper should be consulted, he estimated the volume occupied by the non-liquid phase of the cell as 64.4 per cent (this includes the relatively thick cellulose wall of the yeast cell). In the experiment given below the concentration of the external medium with which the cells are in equilibrium is expressed as relative pressure (1.0 corresponding to a solution of NaCl containing 0.6 per cent at the beginning and 0.55 per cent at the end of the experiment).

P	$(V - b)$	$P(V - b)$
1.0	8.4	8.4
1.17	7.4	8.6
1.45	5.8	8.4
1.64	5.2	8.5
1.74	4.8	8.4

It is seen that the product $P(V\text{-}b)$ is constant.

Summary. It may be concluded that under optimal conditions the osmotic volume changes of cells follow closely the course predicted by the law of Boyle-van't Hoff, provided that cell volume is corrected for osmotically "inactive" material and the semipermeability of the cell remains intact under the experimental conditions.

C. *Applicability of the law of Charles-van't Hoff to cells.* Charles' law states that the osmotic pressure of dilute solutions is proportional to the absolute temperature.

1. If the cell were an ideal osmotic system it would be expected that a solution isotonic with the cell contents at one temperature would also be isotonic with the cell contents at another temperature. In other words, if a cell is in osmotic equilibrium with a certain solution at a given temperature, it would, according to Charles' law, remain in osmotic equilibrium (as measured by cell volume) with the same solution at any other temperature. This indeed appeared to be the case in the experiments of Hamburger (37), who found that erythrocytes brought into equilibrium with dilute solutions of various substances at 14°C. did not alter in volume when the temperature was lowered to 0° or raised to 34°C. Hamburger's experiments were cited by van't Hoff (119) as supporting the validity of the law of Charles as applied to solutions. However, later investigators using more refined methods did not obtain so simple a relation between temperature and osmotic pressure of cells and me-

dium. Thus, Jarisch (59), using several species of erythrocytes obtained a definite and graded decrease in the degree of osmotic hemolysis with increase in temperature.

Similarly, Jacobs (57), using an exceedingly sensitive method for determining that volume of the swollen erythrocyte at which hemoglobin escapes from a given percentage of cells, and holding constant the pH and oxygen tension of the medium (also factors which influence the degree of hemolysis) found likewise a clear-cut decrease in the degree of hemolysis with rise of temperature. This is well shown by the following figures, taken from a more extensive series. (The figures are taken from graph 4 of Jacobs' paper; the degree of hemolysis is 60 per cent, i.e., hemoglobin has been liberated completely from 60 per cent of the cells.)

TEMPERATURE	CONCENTRATION
°C.	*mols per liter*
0	0.103
10	0.095
20	0.088
30	0.083
40	0.080

It is seen that a change in temperature of 10°C. is exactly balanced by change in concentration of from 0.003 M. to 0.008 M.; for example, at 10° the osmotic pressure of the solution which causes hemolysis of 60 per cent of the cells is about 0.007 M. greater than at 20°. In other words, as the temperature increases the osmotic pressure of the corpuscle decreases. Since by Jacobs' method the osmotic effects of solutions differing from one another by 0.0002 M. or less may be distinguished, it follows that a change in temperature which can produce a measurable effect is approximately 0.5°C. Jacobs shows that these experimental facts can be accounted for by the osmotic effects which temperature produces in the base-binding power of hemoglobin, namely, with increasing temperature more base is bound to hemoglobin and hence the osmolar concentration decreases within the cell. This would lead to a shift in water and a decrease in cell volume. He derives equations by which the theoretical volume changes due to changes in the base-binding power of the hemoglobin may be estimated, and he finds that the observed effects are of the order of magnitude predicted by these equations. It may, then, be assumed that the effect of temperature is pri-

marily an osmotic one, and that the experimental facts are subject to explanation based upon the known physico-chemical properties of the erythrocyte.

2. Fauré-Fremiet (24) studied the effect of temperature on the volume of unfertilized eggs of the sea urchin, *Sabellaria alveolata*, in sea water.

He found that between 18° and 25°, i.e., at normal temperatures, cell volume is constant. At lower temperatures the egg increases in volume, while elevation of temperature above 25° brings about shrinking. At 0°, cell volume is increased 3 per cent, at 34° is decreased 7 per cent from its normal value.

Fauré-Fremiet's measurements were as follows:

TEMPERATURE	VOLUME
°C.	cm^3.
0	15.61×10^{-8}
14	15.38
18	15.15
21	15.15
25	15.15
30	14.58
34	14.13

Essentially the same effect of temperature was found by Ephrussi (22) in the case of eggs of the sea urchin, *Paracentrotus lividus*.

It is very probably that these experimental facts may be accounted for along lines similar to those stated for erythrocytes, for the proteins of all cells have the ability to bind base.

Summary. The law of Charles-van't Hoff as applied to cells has been investigated by measuring the volume of cells at different temperatures. It has been found that with increase of temperature the volume of the cell decreases. This apparent deviation from Charles' law may be accounted for, primarily, by the effect of temperature on base-binding ability of the cell proteins.

II. THE KINETICS OF OSMOSIS. In the preceding section we have been mainly concerned with the application to cells of the laws which govern osmotic equilibrium. When two solutions *not* in osmotic equilibrium are separated by a semipermeable membrane, the transfer of water from one solution to the other will be governed by the laws of diffusion. These laws state that the rate of transfer will depend on the difference in osmotic pressure of the two solutions, on the absolute temperature, and on the area and thickness of the membrane; their quantitative

expression will include a constant characteristic of the nature of the membrane. This constant is concerned with the rate with which water can pass through a given quantity of membrane, under given conditions of osmotic pressure and of temperature; it may be termed the permeability of the membrane to water.

With non-living systems technical difficulties have limited the study of the kinetics of osmosis (94, 95); with certain marine ova, however, it is possible to follow the rate of flow from beginning to end of the process and so to determine to what extent the laws of diffusion are obeyed by living material.

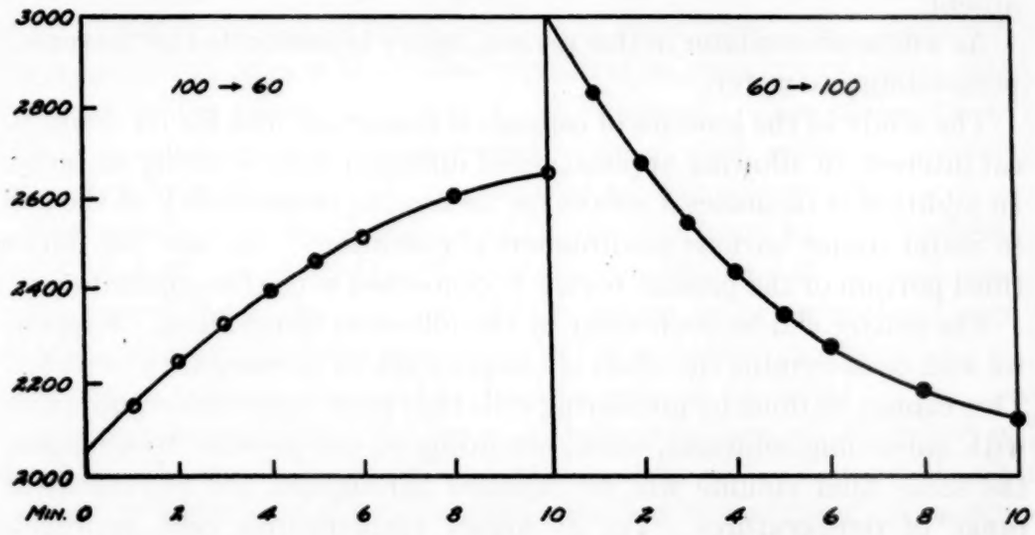

Fig. 2. Graphs illustrating the course of swelling and of shrinking of living uninjured cells in anisotonic solutions at constant temperature (18°C.). The mean volume of six cells is plotted against time. The left graph shows the course of swelling (endosmosis of water); cells removed from their natural medium (100 per cent sea water) were placed in 60 per cent sea water, and measured at intervals of one minute. In the right graph, shrinking (exosmosis of water) is illustrated. Cells were first allowed to swell in 60 per cent sea water for forty-five minutes; the swollen cells were then transferred to 100 per cent sea water, and measured at intervals of one minute.

1. For this type of experiment, sea urchin eggs have special advantages. As has been already stated, their spherical shape allows direct measurement of the volume of water entering or leaving the cell. They maintain their shape over a wide range of osmotic pressures. Their permeability to water is relatively low so that half an hour or even much longer may be required for the cell to come into osmotic equilibrium with an anisotonic solution. Consequently volumes may readily be

measured at minute intervals. When these volumes are plotted against time a curve is obtained which describes the course of swelling or shrinking in anisotonic solutions. Such curves are given in the accompanying graphs (fig. 2).

In the case of any osmometer it is essential to know whether the membrane has suffered injury either before or during an experiment. Injury may readily be determined in the case of this material by returning the ova to ordinary sea water at the end of an experiment and then adding sperm. In the absence of injury, ova then undergo normal cleavage, whereas if injury or death of cells has occurred, cleavage is atypical or absent.

As will be shown later in this review, injury is manifested by increased permeability to water.

The study of the kinetics of osmosis is important first for its theoretical interest, in allowing application of diffusion laws to living material. In addition it furnishes a means for measuring permeability of the cell to water under various environmental conditions. In fact the entire third portion of the present review is concerned with this application.

The matter will be made clear by the following illustration. Suppose we wish to determine the effect of temperature on permeability to water. This cannot be done by measuring cells that have come into equilibrium with anisotonic solutions, since, according to our present information, the same final volume will be attained throughout the physiological range of temperatures. Yet at higher temperatures cells approach their equilibrium volume much more rapidly than at lower temperatures. It is therefore the rate of flow that must be measured; it is the *kinetic* method that must be employed.

With this end in view, several theories of osmotic kinetics have been developed and applied to marine ova.

The first was that of Lillie (70) who worked with the eggs of the sea urchin, *Arbacia punctulata*. He wished to determine whether fertilized eggs are more permeable to water than are unfertilized ones.

The ova were suspended in a solution consisting of 40 parts of sea water and 60 parts of tap water. A drop of this suspension was placed on a glass slide and observed through a microscope provided with an ocular micrometer. The diameter of spherical cells was measured each minute until it became constant.

The swelling process was found to follow an equation similar to the one used to describe the course of a unimolecular chemical reaction. Lillie's equation was

$$K = \frac{1}{t} \ln \frac{V_e - V_o}{V_e - V_t} \quad (1)$$

in which V_e,V_o and V_t are volumes at equilibrium, at the first instant and at time t, respectively, and K is a factor expressing the permeability of the cell to water. V_o was found by measuring a number of cells before beginning an experiment, V_e was the final volume reached by the cells and V_t was found for each minute of the swelling process.

This equation was found to fit the data satisfactorily when either fertilized or unfertilized eggs were used. Values of K determined at successive minutes of the swelling process showed no drift in either case, being higher in the case of fertilized than of unfertilized ova. However, when applied to eggs with artificial fertilization membranes, there was a steady falling off in the value of the constant. This indicated, in Lillie's opinion, imperfect semipermeability of such cells, an opinion supported by work which will be discussed later in this review.

The equation (1) developed by Lillie was later used by McCutcheon and Lucké (87) to study the effect of temperature and of the salt concentration of the medium on the rate of osmosis, using unfertilized eggs of the sea-urchin, *Arbacia punctulata*. The equation gave good fits at a variety of temperatures and for various osmotic pressures.

In a subsequent paper the same authors investigated the course of *exosmosis* of water from unfertilized Arbacia eggs (88). Eggs previously swollen in hypotonic solutions were returned to ordinary sea water. Under this condition water leaves the cell under the driving force of osmotic pressure, the cell shrinking until it regains its original volume (82). Equation (1) was found to fit the course of shrinking also.

Up to this point equation (1) had proved satisfactory, allowing comparison of permeabilities when the osmotic pressure of the medium was held constant and some other factor, such as temperature, was varied.

When, however, the osmotic pressure was varied, analysis by means of equation (1) gave rather misleading results. In a paper by Lucké and McCutcheon (83) permeabilities to water were compared when cells were caused to swell or shrink in sea water dilutions of different osmotic pressures, temperature being held constant. Values of K were then found to vary greatly at different osmotic pressures, apparently indicating that the cell is more permeable to water at higher salt concentrations of the medium than at lower ones.

Such a conclusion is justified when permeability is defined as in equation (1). But, as pointed out by Northrop (95), this definition of

permeability is devoid of physical significance, equation (1) being purely empirical.

Northrop then proceeded to develop two equations by means of which permeability could be measured in physical units, e.g., the quantity of water passing across the membrane per unit time under a given osmotic driving force.

The point of departure of Northrop's treatment, as well as of equations developed by Lucké, Hartline and McCutcheon (86), is the assumption that the volume of water passing the membrane per unit time is directly proportional to the difference in pressure between the cell contents and the medium, to the area of the membrane and inversely to the thickness of the membrane. Altering Northrop's symbols slightly to conform to those used by the present writers, we write

$$\frac{dV}{dt} = \frac{S}{h}(P - P_{ex}) \tag{2}$$

in which $\frac{dV}{dt}$ is change in volume per unit time, S is area of cell surface, h is thickness of cell membrane, P is osmotic pressure of cell contents and P_{ex} that of the external solution.

The problem is most simply treated by assuming that S and h are effectively constant or vary only to an insignificant degree in the course of swelling or shrinking. In this case, K, S and h may be combined in a new constant, C, and we write:

$$\frac{dV}{dt} = C(P - P_{ex}) \tag{3}$$

in which C is a factor of proportionality expressing the permeability of the cell to water.

This equation cannot be integrated as it stands. However, the necessary relations between V and P are given by the equation.

$$PV = P_oV_o,$$

which has been developed in a previous section of this review. P_{ex} is a constant in the integration; for the sake of symmetry, however, we shall introduce the constant V_e, volume at equilibrium, given by the equation

$$P_{ex}V_e = P_oV_o$$

making the necessary substitutions in (3) we obtain

$$\frac{dV}{dt} = C \cdot P_o V_o \left(\frac{1}{V} - \frac{1}{V_e}\right).$$

of which the integrated form is

$$C \cdot P_o V_o \cdot t = V_e^2 \ln \frac{V_o - V_e}{V - V_e} + V_e (V_o - V) \tag{3a}$$

Northrop found that this equation satisfactorily expresses the rate of osmosis across a collodion membrane. He recalculated Lillie's data on the swelling of fertilized and artificially activated *Arbacia* eggs, and found the equation fitted. Indeed equation (3a) gave a better fit than did the unimolecular equation (1). With unfertilized eggs (data of Lucké and McCutcheon), the fit was not satisfactory, the value of C increasing during the course of swelling. This result led Northrop to develop his second treatment.

This was based on equation (2), in which area and thickness of membrane are assumed to vary. The volume, m, of the membrane is the product of its area, S, and its thickness, h. This volume is assumed constant. We have

$$h = \frac{m}{S}$$

Substituting in equation (2) we have

$$\frac{dV}{dt} = C_2 S^2 (P - P_{ex}) \tag{4}$$

in which C_2 is a new factor of proportionality.

Alternatively, water may be assumed to flow only through pores in the cell surface, which undergo change in size. The rate is then proportional to the fourth power of the radius or the square of surface of the pores, a consideration which leads again to equation (4).

Expressing this equation in terms of volume, we obtain:

$$\frac{dV}{dt} = C_2 (36\pi)^{\frac{1}{3}} \cdot V^{\frac{4}{3}} \cdot P_o V_o \left(\frac{1}{V} - \frac{1}{V_e}\right)$$

of which the integrated form is

$$C_2 (36\pi)^{\frac{1}{3}} P_o V_o \cdot t = V_e^{\frac{4}{3}} \left[\tfrac{1}{2} \ln \frac{V_e^{\frac{2}{3}} + (V_e V)^{\frac{1}{3}} + V^{\frac{2}{3}}}{(V_e^{\frac{1}{3}} - V^{\frac{1}{3}})^2} - \sqrt{3} \tan^{-1} \frac{2 V^{\frac{1}{3}} + V_e^{\frac{1}{3}}}{\sqrt{3}\, V_e^{\frac{1}{3}}} \right]_{V_t = 0}^{V} \tag{4a}$$

This equation was applied by Northrop to the data of the present writers (83, 87) (unfertilized *Arbacia* eggs) and a rather satisfactory fit obtained. The effect of salt concentration of the medium, indicated by the unimolecular equation (1) was greatly reduced when equation (4 a) was used, although, with the data available to him, there was still an appreciable effect.

This result led Lucké, Hartline and McCutcheon (86) to reinvestigate the effect of salt concentration of the medium with a more extensive series of experiments. Since the empirical nature of equation (1) had been pointed out prior to the appearance of Northrop's paper, an equation whose derivation differs somewhat from those of Northrop had already been developed. This equation, as well as Northrop's equation (4 a) were applied to the new data.

It is convenient at this point to state the assumptions which were made in formulating the new treatment. Of these, all but the last apply equally to Northrop's equations.

1. The *Arbacia* egg consists of a solution of osmotically active substances, surrounded by a semipermeable membrane.

The fact that a fraction of cell volume is occupied by osmotically inactive material is neglected for the moment for the sake of simplicity, but will be introduced later as a correction.

2. The aqueous systems concerned obey the laws of ideal dilute solutions.

3. Only osmotic forces are concerned in the passage of water across the surface of the cell.

4. Resistance to the diffusion of water is confined to the surface of the egg.

5. Resistance to the diffusion of water per unit area of cell surface is constant.

The present osmotic theory states that the rate of transfer of water into or out of the egg will be proportional to the area of its surface and to the difference in osmotic pressure between the interior and the external medium. The resulting equation differs from equation (2) only in regarding the thickness of the membrane as constant. We write

$$\frac{dV}{dt} = k \cdot S\,(P - P_{ex}) \tag{5}$$

in which the symbols have the same significance as before. This definition of permeability (like Northrop's definitions) is seen to possess definite physical meaning, viz., the number of cubic micra of water en-

tering the cell per minute per unit area of membrane, per atmosphere of difference in osmotic pressure between interior and external medium. Making the substitutions necessary before integration, we write

$$\frac{dV}{dt} = k\,(36\,\pi)^{\frac{1}{3}} \cdot V^{\frac{2}{3}} \cdot P_o V_o \left(\frac{1}{V} - \frac{1}{V_e}\right)$$

which, when integrated, becomes

$$k(36\,\pi)^{\frac{1}{3}} \cdot P_o V_o \cdot t = V_e\left[V_e^{\frac{1}{3}}\left(\tfrac{1}{2}\ln\frac{V_e^{\frac{2}{3}} + (V_e V)^{\frac{1}{3}} + V^{\frac{2}{3}}}{(V_e^{\frac{1}{3}} - V^{\frac{1}{3}})^2} + \sqrt{3}\tan^{-1}\frac{2\,V^{\frac{1}{3}} + V_e^{\frac{1}{3}}}{\sqrt{3}\,V_e^{\frac{1}{3}}}\right) - 3\,V^{\frac{1}{3}}\right]_{V_t=0}^{V} \qquad (5a)$$

Equations (4 a) and (5 a) were tested in a series of 10 experiments, in which cells were caused to swell in various dilutions of sea water; also in a number of experiments in which cells previously swollen were caused to shrink in ordinary sea water.

The accompanying graph (fig. 3) represents the course of swelling in 3 experiments calculated by equation (5 a).

$$\frac{1}{(36\,\pi)^{\frac{1}{3}} \cdot P_o V_o} \cdot f(V,\, V_e)$$

(in which $f(V, V_e)$ stands for the right side of equation (5 a)) is plotted against time and a linear fit is obtained. The slope of the line gives the value of k, permeability to water.

The next graph (fig. 4) represents a shrinking experiment. Cells previously swollen in 60 per cent sea water have been returned to ordinary sea water and the course of shrinking observed and computed by (5 a). The fit is again linear. The temperature was in each case 15°C.

The following conclusions were drawn from these experiments:

(a) Equation (5 a) fits the data for both swelling and shrinking, at least during the early part of these processes. The graph shows deviation of the later points in 20 per cent sea water; this deviation is believed to represent a true increase in permeability to water associated with injury to the cell. Deviation in the opposite direction is observed in 60 per cent sea water; this means that the equation does not fit the data during the latter part of the swelling process.

(b) Equation (4 a) fits the data just as well as does equation (5 a). The equations are so similar that it appears impossible to choose between them experimentally, except in extremes of osmotic pressure that cannot be employed or by measurements made so close to equilibrium

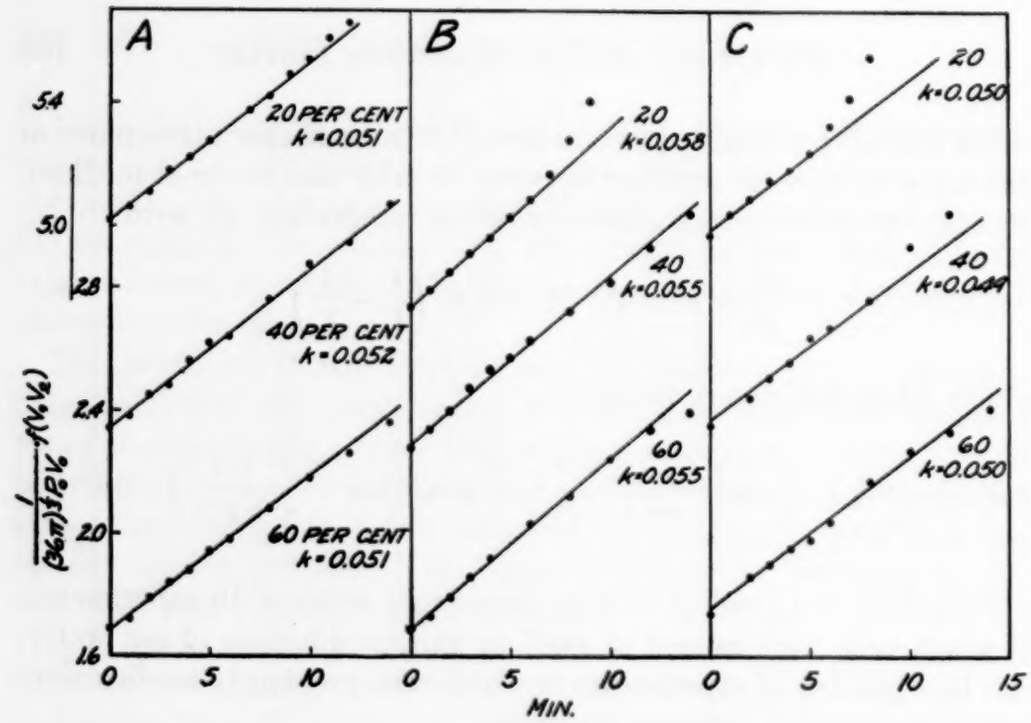
A
B
C
20 PER CENT
k = 0.051
40 PER CENT
k = 0.052
60 PER CENT
k = 0.051
20
k = 0.058
40
k = 0.055
60
k = 0.055
20
k = 0.050
40
k = 0.049
60
k = 0.050
5.4
5.0
2.8
2.4
2.0
1.6
0
5
10
15
MIN.

Fig. 3

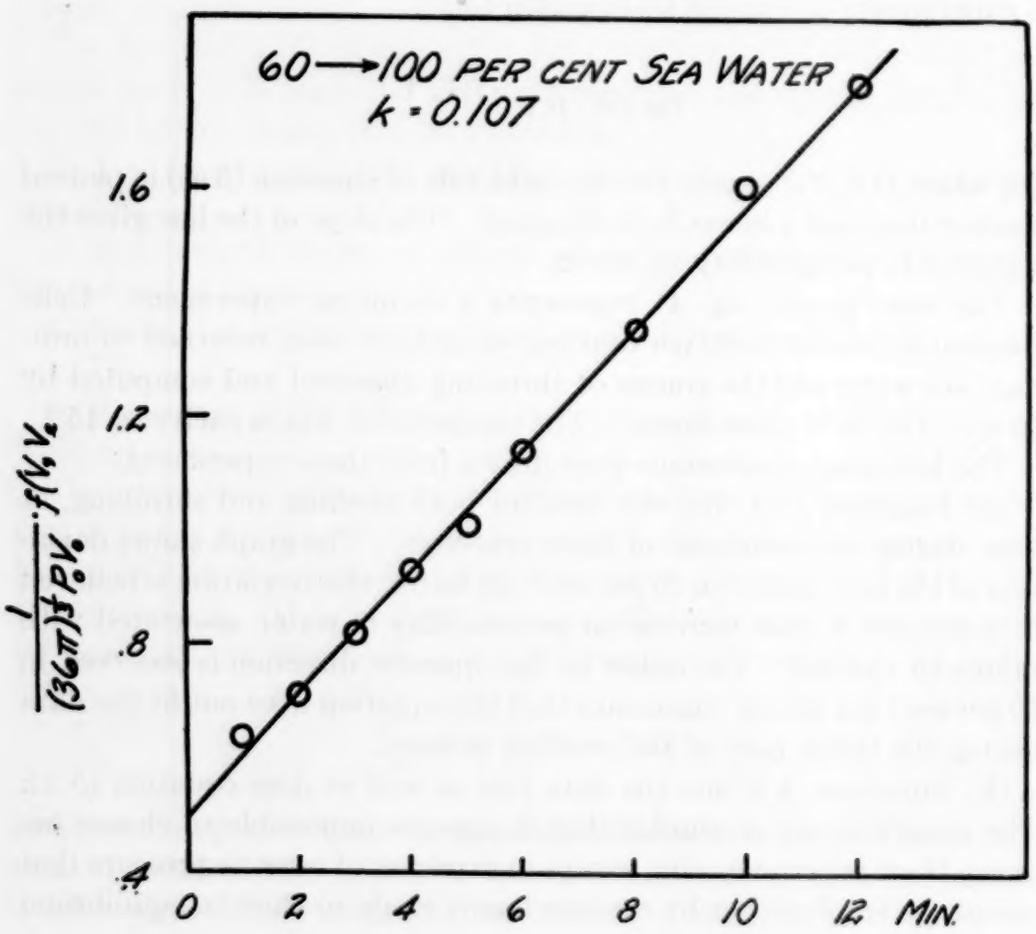
60 → 100 PER CENT SEA WATER
k = 0.107
1.6
1.2
.8
.4
0
2
4
6
8
10
12
MIN.

Fig. 4

that the experimental error is greater than differences between the equations.

It is therefore a matter of indifference which equation is selected.

(c) There is no effect of salt concentration of the medium on k or C_2. The values of these proportionality factors show no drift over the concentration range employed (4.4 to 13.2 atmospheres; on the basis of other experiments, from 4.4 to 17.6 atmospheres). This means that permeability of the cell to water is independent of the osmotic pressure of the outside solution. It was the principal aim of these experiments to settle this point. The concentration effect previously found by Lucké and McCutcheon (83) was due to the empirical nature of the equation used. The slight concentration effect reported by Northrop was doubtless due to the scantiness of the data available at that time.

(d) In shrinking, permeability as defined in (4) and (5) is invariably greater than in swelling. More accurately stated, this is true when shrinking from hypotonic to isotonic sea water is compared with swelling from isotonic to hypotonic sea water, temperature being constant. This relation is brought out in the accompanying table, which gives values of k_5 (i.e., the proportionality factor of equation (5 a)) for swelling in 60 per cent sea water and for shrinking in 100 per cent sea water at several temperatures.

TEMPERATURE	SWELLING		SHRINKING	
	k_5	k_6	k_5	k_6
12°	0.043	0.047	0.067	0.059
15°	0.051	0.059	0.074	0.067
18°	0.071	0.079	0.098	0.086
21°	0.107	0.111	0.142	0.128
24°	0.135	0.156	0.175	0.157

A large number of other experiments yielded a similar result in every case.

This unexpected and interesting relation at once suggests that equation (5 a) is incomplete as it stands, that some additional factor has been left out of account which operates differently in swelling and shrinking. One such additional factor is not far to seek.

Early in this discussion it was deduced from equilibrium data that the cell swells not as if its volume were V, but as if it were V-b, in which b is osmotically inactive substance, constituting about 12 per cent of the cell volume. This correction must be introduced in the kinetic equations, and it will be found to affect swelling and shrinking differently.

Making the necessary substitutions in equation (5) and integrating, we obtain

$$k\,(36\,\pi)^{\frac{1}{3}} \cdot P_o(V_o - b)\quad t = (V_e - b)\left[\left(1 - \frac{b}{V_e}\right) \cdot V_e^{\frac{1}{3}}\left(\tfrac{1}{2}\ln \frac{V_e^{\frac{2}{3}} + (V_e V)^{\frac{1}{3}} + V^{\frac{2}{3}}}{(V_e^{\frac{1}{3}} - V^{\frac{1}{3}})^2}\right.\right. \\ \left.\left. + \sqrt{3}\tan^{-1}\frac{2\,V^{\frac{1}{3}} + V_e^{\frac{1}{3}}}{\sqrt{3}\,V_e^{\frac{1}{3}}}\right) - 3\,V^{\frac{1}{3}}\right]_{V_{t=0}}^{V} \tag{6}$$

Equation (6) has been found to fit the data, if anything, a little better than (5 a). In the preceding table, permeability as recalculated by (6) has been expressed as values of k_6. These values may be compared with those of k_5. It is seen that the new equation slightly increases permeability values in swelling but considerably decreases them in shrinking. Thus values for swelling and shrinking are brought more closely together.

When applied to a number of experiments it has been found that the average ratio of shrinking to swelling as calculated by equation (5 a) is 1.51; when calculated by equation (6), the average ratio is 1.25. Thus the difference between swelling and shrinking is reduced by one-half.

But a significant difference still remains: water leaves the cell more readily than it enters. Consideration of the forces such as elasticity and surface tension, and of mechanisms within the cell which might bring about this result, will be made the subject of a future paper.[9a]

The present status of the kinetics of osmosis may be summarized as follows:

Attempts have been made by Northrop and by Lucké, Hartline and McCutcheon (86) to account for the course of swelling and shrinking of eggs of *Arbacia punctulata* in anisotonic solutions, by means of the laws governing osmosis and diffusion. The method employed has been to compute permeability of the cell to water, as measured by the rate of volume change per unit of cell surface per unit of osmotic pressure outstanding between the cell and its medium.

[9a] Recent experiments of E. N. Harvey (Biol. Bull. 1932, lxi, 273), and K. S. Cole (personal communication) have shown that in the case of the unfertilized egg of *Arbacia punctulata* surface forces (including elastic forces) are of such a magnitude that the internal hydrostatic pressure produced by them is less than $\frac{1}{2000}$ of an atmosphere. Pressures of this magnitude are of course quite negligible as compared with osmotic pressures involved in the experiments described above.

See also the recent paper by T. Kamada and T. Yamamoto (Journ. of the Faculty of Science, Imp. Univ. of Tokyo, Sect. IV, 1931, II, 357).

Permeability to water as thus defined is approximately constant during swelling and shrinking, at least for the first several minutes of the process.

Permeability is found to be independent of the osmotic pressure of the solution in which cells are swelling.

Water is found to leave cells more readily than it enters, that is, permeability is greater during exosmosis than during endosmosis.

In a later section of this review these methods will be found useful for determining the effect of various environmental factors on the permeability of the cell to water.

The effect of the salt concentration of the medium on permeability to water has been determined for a number of marine ova by Leitch (69). He investigated the following species obtained on the Pacific Coast (California): *Dendraster excentricus* (sand dollar), *Patiria miniata* and *Pisaster ochraceous* (starfish), *Strongylocentrotus franciscanus* and *S. purpuratus* (sea urchins) and *Urechis* (an annelid). Eggs were caused to swell in several dilutions of sea water and permeability determined (at the third minute) by means of equation (5). The volume per cent of osmotically inactive material was assumed to be 15.

As seen in the accompanying table the results in some cases agree with those obtained from *Arbacia punctulata* (given above) in showing no definite variation in permeability with the osmotic pressure of the medium, while in other species there is a drift in values suggesting that permeability increases as the external pressure is decreased. These ova are all more freely permeable to water than are those of *Arbacia punctulata*.

	SEA WATER			TEMPERATURE
	40 per cent	50 per cent	60 per cent	
				°C.
Dendraster exc.	0.266	0.234	0.204	20–22
Dendraster (jelly removed)	0.197	0.228	0.195	20–22
Patiria min.		0.226	0.185	20–22
Pisaster och.	0.397	0.389	0.442	20–22
Strong. fran.	0.141	0.133	0.110	17–19
Strong. purp.	0.097	0.090	0.091	17–19
Urechis	0.147	0.138	0.130	20–22

(2) Experiments with erythrocytes. The kinetics of osmotic swelling of erythrocytes have been studied by Jacobs (53, 58). We have seen that in the case of the spherical echinoderm egg cell the surface area

changes as the volume increases or decreases during osmotic swelling or shrinking. In the erythrocyte, however, which is normally biconcave in shape, considerable increase in volume may take place before its surface begins to be altered to a significant degree.

Jacobs therefore makes the simplifying assumption that the area of the erythrocyte does not change during the process of swelling. He expresses the rate of increase in volume by the equation

$$\frac{dV}{dt} = k \cdot A \ (p - P)$$

where A is the area of the erythrocyte, V its volume at time t, p its osmotic pressure, P the constant and known osmotic pressure of the surrounding solution, and k the permeability constant.

On integration he obtains

$$k = \frac{P_o V_o}{P^2 A t} \ln \frac{P_o V_o - P V_o}{P_o V_o - P V} - \frac{V - V_o}{P A t}$$

where P_o and V_o are the initial osmotic pressure and volume of the erythrocyte, respectively. When the external medium is water, and P is therefore zero, the equation becomes

$$k = \frac{V^2 - V_o^2}{2\, p_o V_o\, A t}$$

The permeability constant, k, has the same meaning as in the case of *Arbacia*; it expresses the amount of water which will enter the erythrocyte in unit time through unit area under unit difference of osmotic pressure.[10] Unfortunately, as Jacobs points out, the existing data for the volumes and surfaces of erythrocytes of different species are far from being exact; even so, the equation permits at least a determination of the magnitude of the value of the permeability to water for erythrocytes.

III. *The permeability of the cell to water and factors which modify it.* In the preceding section on the kinetics of osmosis a quantity has been introduced characteristic of the nature of the cell membrane. We have termed this the permeability of the cell to water. It has been indicated how this quantity may be computed. In the case of *Arbacia* we may

[10] The data with which these equations have been tested will be published shortly (personal communication).

calculate the permeability by means of the integrated equations (4 a), (5 a), or (6). Alternatively, if the rate of change of volume $\frac{dV}{dt}$ be measured directly from the graph of the swelling or shrinking curve, the permeability may readily be computed by the differential equation (5). For details the original papers should be consulted.

We shall now concern ourselves with various factors which modify the permeability of the cell.

A. *The effect of temperature.* It is well known that most biological processes in non-living material proceed more rapidly at higher temperatures than at lower. To this rule the rate of passage of water across the cell surface is no exception.

With non-living material, chemical processes are frequently more affected by temperature than are such physical processes as diffusion. A convenient way of measuring the temperature effect is to determine the ratio of the rates of a process at 2 temperatures, 10°C. apart. This ratio is known as Q_{10}.[11]

The value of Q_{10} for most chemical processses is 2 or above, for simple diffusion, about 1.3.

Another method of computing temperature effect has been used by Crozier and other biologists (11). This is the Arrhenius equation

$$\ln \frac{k_1}{k_2} = \frac{q}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right)$$

in which k_1 and k_2 are velocity constants at different temperatures, q (often written μ) is heat of activation, R is the gas constant and T the absolute temperature. The advantage of this method is that the value of the temperature characteristic, μ, varies somewhat less with temperature than does Q_{10}. For chemical reactions the value of μ is usually high, from 10,000 to 30,000 calories.

Although the temperature coefficient is frequently high in biological processes, this fact may not justify the conclusion that chemical processes are concerned. The rate of osmosis of water is a case in point. Values of Q_{10} of 2 or above have frequently been reported, but that any

[11] A convenient form of the equation for determining this value is,

$$\log Q_{10} = \frac{\log K_1 - \log K_2}{t_1 - t_2} \times 10$$

in which K_1 is the rate at the higher, K_2 at the lower temperature, and $t_1 - t_2$ is the difference in temperature.

chemical process is involved has not been established and is indeed improbable.

(a) With plant material, the subject of temperature effect on permeability to water has been so carefully reviewed by Stiles (113) that only brief reference is necessary in this article. As pointed out by Stiles, much of the earlier work was affected by errors of one kind or another so that the only conclusion that can be drawn is that the process proceeds more rapidly at higher than at lower temperatures. Excellent work has been done on absorption of water by such storage tissues as potato and seeds. Here, however, the process appears to be more complex and to involve hydration of colloids. It is not probable that the chief resistance to the passage of water is encountered at the cell surface, so that conclusions as to the effect of temperature on permeability of the cell to water cannot be drawn with any confidence. Indeed, Northrop (95) showed that the rate of swelling of potato and carrot, as reported by Stiles and Jorgensen (111), follows the kinetics of swelling displayed by gelatin rather than by living cells. The situation with storage tissues has been reviewed by Stiles (113), Shull (109) and by Denny (13 a).

(b) With plant material, the most reliable work on temperature in relation to permeability of the cell to water is by Delf (13). Her materials were the leaf of onion and the scape of dandelion. Solutions of cane-sugar were allowed to flow through these hollow organs and the rate of linear shrinkage determined at several temperatures. She computed the following temperature coefficients:

TEMPERATURE	ONION Q_{10}	DANDELION Q_{10}
°C.		
5–15	1.4	
10–20	1.5	2.3
15–25	2.0	3.3
20–30	2.6	3.8
25–35	2.9	3.0
30–40	3.0	2.6

It is seen that most of these values are high, indicating a great effect of temperature on exosmosis. The only objection that can be raised to this work is that tissues rather than isolated cells were used. It may be questioned whether permeability of the cell to water was the only factor involved in the rate of linear shrinkage.

(c) Turning now to experiments with animal material, it was reported by Bialaszewicz (8) that unfertilized frog eggs took up water five times as rapidly at 20° as at 10°. His method is open to the objection that he compared amounts of water taken up in equal times instead of the times required to produce a certain fraction of the total volume change. Therefore the ratio 5 would not be expected to hold good for other parts of the swelling curves.

(d) The effect of temperature on permeability of unfertilized sea urchin eggs (*Arbacia punctulata*) has been studied by the present writers (86, 87, 88). The temperature effect was high,[11a] both in endosmosis and exosmosis of water, values of μ lying between 13,000 and 17,000. The results of a representative experiment follow (for original data see (86)). Cells were swollen in 60 per cent sea water and then were shrunken in 100 per cent sea water. Permeability calculated by equation (6) is given in column 1, Q_{10} in column 2.

	TEMPERATURE	1	2	3	4
		k	Q_{10}	$k \cdot \eta$	Q_{10}
	°C.				
Course of swelling	12	0.047	2.1	0.058	1.6
	15	0.059	2.6	0.067	2.2
	18	0.079	3.1	0.085	2.3
	21	0.111	3.1	0.109	2.4
	24	0.156		0.142	
Course of shrinking	12	0.059	1.5	0.073	1.1
	15	0.067	2.3	0.076	1.9
	18	0.086	3.8	0.092	2.8
	21	0.128	2.0	0.125	1.6
	24	0.157		0.143	

This temperature effect probably contains at least 3 components, only one of which can be regarded as change in permeability to water. These factors are as follows:

(1) The osmotic pressure of an ideal solution varies with absolute temperature. As already pointed out, if cell contents and medium were both ideal solutions, they would be equally affected by change in temperature and the rate of flow would not be altered. Since in fact we

[11a] The temperature coefficient of the permeability constant of fertilized *Arbacia* eggs has recently been found by us to be even higher than that of unfertilized eggs (unpublished experiments).

are not dealing with ideal solutions and since the composition of cell and medium is different, it is to be expected that their osmotic pressures will be differently affected by temperatures. However, according to the work of Fauré-Fremiet (24) already referred to, the osmotic pressure of the eggs of *Sabellaria alveolata* is altered only slightly in the temperature range employed in the work of the present writers. Until data comparable to Fauré-Fremiet's are available for *Arbacia*, the effect of temperature on the osmotic pressure of cell and medium had best not be discussed; in any event it is presumably small enough to be safely neglected.

(2) It was pointed out by Northrop (94, 95) that "if water is supposed to flow through holes in the membrane," the permeability factor, k, "contains the viscosity of water and should be corrected for the viscosity of water before comparing the values at different temperatures." At present it appears uncertain whether water does in fact pass through definite pores or merely through intermolecular spaces. The second possibility is identical with the theory that water enters the cell by being dissolved in the membrane. However, according to Bigelow, such intermolecular spaces would, like pores, act as capillary tubes, so that water flowing through them would be subject to Poisseuille's law. This law contains a factor for viscosity of water. It therefore seems reasonable to correct temperature effect on osmosis for viscosity of water.

Northrop did this (95), using early data of the present writers, and concluded that the temperature effect was largely explained by changes in viscosity of water. There remained, however, part of the temperature effect which cannot be so explained. This was shown more convincingly by later experiments such as the one recorded in the preceding table. In column 3 of this table, the permeability factor has been corrected for viscosity of water η, and from the values so obtained, temperature coefficients in column 4 are computed. Values of Q_{10} so corrected are seen to vary widely.

(3) There remains, then, an effect of temperature on osmosis which apparently cannot be explained by changes in the solutions concerned and which therefore, by exclusion, must be attributed to change in permeability of the cell surface. The nature of this change in permeability is quite unknown; indeed it cannot at present be stated, on the basis of these experiments, whether the alteration in permeability is reversible or irreversible.[11b]

[11b] Recent unpublished experiments of the present writers show that the temperature effect in the case of unfertilized *Arbacia* eggs is in fact reversible. For

(e) Jacobs (54, 58), working with ox erythrocytes, found a low temperature effect on the swelling of cells in hypotonic NaCl and dextrose solutions. The value of Q_{10} was about 1.25, which is the order of magnitude of simple diffusion processes.

Effect of temperature on the rate of osmotic hemolysis. Erythrocytes of the ox. Time in seconds for 75 per cent hemolysis

TEMPERATURE	WATER		0.02 M NaCl	
	Time	Q_{10}	Time	Q_{10}
0°	2.4		6.5	
		1.26		1.25
10°	1.9		5.2	
		1.36		1.16
20°	1.4		4.5	
		1.27		1.33
30°	1.1		3.4	
		1.22		1.13
30°	0.9		3.0	

Jacobs points out two sources of error in determining the effect of temperature on permeability by the hemolytic method. The volume which the cell attains before undergoing hemolysis varies, but Jacobs found that when large numbers of cells are used under rigidly controlled conditions, the mean volume at which a certain percentage of cells disappear is definite. He computed permeability by determining the times required at different temperatures for 75 per cent of cells to undergo hemolysis.

This means of computing permeability is valid under certain conditions. These conditions are that 1, the course of swelling at the various temperatures employed shall follow the same equation; 2, the initial volumes of the cells shall be the same; 3, the final volumes of the cells at equilibrium shall also be the same. The first condition may plausibly be assumed to exist; the second condition is met by using large numbers of cells; but Jacobs found that the third requirement was not met by his material under all conditions; the cells did not reach the same equilibrium volumes at different temperatures in solutions sufficiently concentrated not to hemolyze, and presumably were not approaching the same equilibrium in hemolyzing solutions. On the contrary, he found

example permeability was determined for eggs swollen at 12°C. The cells were then returned to ordinary sea water and, after they had regained their original volume, they were swollen again at 22°C. and permeability determined. Conversely eggs were swollen first at 22° and later at 12°. Permeability values were practically the same for the two lots of cells at a given temperature.

that the higher the temperature, the smaller was the volume at equilibrium. He was thus dealing with two distinct temperature effects which worked in opposite directions. The one effect of increasing temperature was to increase the rate of flow, as is usual in biological processes; the other effect was to decrease the rate of flow by decreasing the distance of the system from equilibrium. According to the conditions chosen, one or the other effect might predominate, the temperature coefficient might be positive or negative.

Once, however, this double effect of temperature was recognized, it was possible to choose conditions that minimized the effect on equilibrium, so that the effect of temperature on water and membrane stood out unobscured. This was accomplished by using only quite dilute solutions in which the cells underwent hemolysis while still far from equilibrium. Then the distance of the system from equilibrium became negligible in its effect on the rate of flow.

Jacobs further pointed out another disadvantage of using solutions of osmotic pressure such as to bring the equilibrium volume close to the critical volume at which hemolysis occurs. A slight change in the pressure of such solutions enormously affects the time required for hemolysis so that determinations become highly unreliable.

Summary. The data just reviewed show, at least for certain of the cells studied, an effect of temperature which is somewhat in excess of what would be expected from the purely physical process of diffusion. It appears that other factors must be taken in consideration. There may even be an effect of temperature on the permeability itself, that is, temperature may cause actual changes in the membrane across which water is diffusing.

B. *The effect of ions on permeability of the cell to water.* It is well known that electrolytes play a prominent rôle in the properties of cells. The effect of low concentrations of electrolytes on permeability to water was reported by the present writers in two papers (89, 84). The material used was the unfertilized *Arbacia* egg. The electrolytes were dissolved in hypotonic solutions of a non-electrolyte, usually dextrose. The concentrations of electrolytes were so small (1×10^{-2} to 1×10^{-6} molal), relative to the concentration of dextrose (usually 3.8×10^{-1} molal), that practically all the osmotic pressure of the solution was exerted by the dextrose. It was therefore possible to vary the electrolyte concentration at will without materially altering the osmotic pressure. Since temperature also was held constant, the only variable was the ionic composition of the medium, the effect of which on permeability

could be directly deduced from the rate with which water entered the cell under the driving force of osmotic pressure.

It is readily seen that if the rate of change of volume, $\frac{dV}{dt}$, be measured directly from the graph of the swelling curve, the permeability, k, may be computed according to equation (5) (cf. section on kinetics of osmosis). As a matter of convenience, permeability was calculated, usually at the 2nd or 3rd minute.

Computation of the permeability to water by the methods already explained affords a quantitative measure of the effect of ions upon this property of the cell.

As a step preliminary to studying the effect of ions it was necessary to determine the permeability to water in the non-electrolyte solutions in which ions were later to be dissolved. Accordingly solutions were prepared of dextrose, saccharose and glycocol, made approximately isotonic with 40 per cent sea water, therefore having an osmotic pressure of about 8.8 atmospheres. In these 3 solutions the rate of osmotic flow was quite rapid; permeability at 12°C. was in the neighborhood of $0.1\mu^3$ per minute as compared with 0.05 in 40 per cent sea water.

(a) The effect of the chlorides of Na and K, and of Ca and Mg was now determined by dissolving these salts in hypotonic dextrose solution. It was found that chlorides of Na and K gave either higher permeability values than did dextrose alone or else values of the same magnitude. Thus in a representative experiment the permeabilities obtained with NaCl and KCl in 0.004 molal concentration were respectively 0.129 and 0.096, as compared with 0.093 in dextrose alone and 0.048 in sea water of the same osmotic pressure.[12]

In contrast with the alkalis, Ca and Mg, in the same concentration, gave values of 0.054 and 0.050 respectively; in other words, they lowered permeability to the value obtained in sea water. These 2 cations were effective in concentrations as low as 0.0005 or even 0.0001 molal.

Chlorides of Li and NH_4 acted as did those of Na and K; no significant differences were found in the effects of cations of the same valence, in these low concentrations.

Definite antagonism could be demonstrated to exist between chlorides of univalent and bivalent cations, in the sense that when both were present in appropriate concentrations, the resulting permeability was intermediate between the values produced by them singly. This is

[12] Lower concentrations of NaCl and KCl produced similar though less definite results; concentrations above 0.01 M. led to rapid cytolysis.

illustrated by an experiment in which 0.0005 M. $CaCl_2$ (in 0.38 M. dextrose) gave a permeability value of 0.047. To this solution were then added different amounts of NaCl, so that the resulting NaCl concentrations were 0.004, 0.008, and 0.016 molal. The corresponding permeability values were 0.050, 0.056, and 0.080. So in this experiment 0.016 M. NaCl was required to overcome definitely the effect of 0.0005 M. CaCl.

These experiments clearly showed that chlorides of univalent cations tend to increase permeability to water, while those of bivalent cations tend to reduce permeability to its value in sea water. These results are in accord with experiments of other investigators who have shown that these salts similarly affect permeability to substances other than water. It is reasonable to infer that the low permeability values obtained in sea water are dependent on the presence of Ca and Mg.

(b) The effect of ions of different valence may be interpreted in either of two ways. The usual interpretation has been that Na and K tend to increase permeability, Ca and Mg to decrease it. An alternative hypothesis is that anions tend to increase permeability, cations to decrease it. The latter explanation is consistent with the experimental results just reviewed, if the assumption is made that when both cation and anion are univalent, the effect of the anion predominates. Thus NaCl tends to increase permeability because (it is assumed) the Cl ion is more effective than the Na ion. But in the case of chlorides of bivalent cations, the cation effect predominates, presumably due to its double charge.

This explanation, based on the supposed antagonism between cation and anion, received impressive support from the next paper to be reviewed, on the effect of valence of ions on cellular permeability to water (84).

In this paper two questions were proposed: do cations tend to lower permeability to water in proportion to their valence and, conversely, do anions tend to increase it in proportion to their valence?

Cation series were investigated first. There were obviously two ways in which this might be done. First, salts containing cations of different valence might be used in equal concentrations. Would the resulting permeability values form a series in which the lowest permeability corresponds to the cation of highest valence?

Without going into details, it may be stated that the answer was negative. No salts were found that produced lower permeability than is found in sea water or in effective concentrations of $CaCl_2$. Apparently

the unfertilized *Arbacia* egg has, in its natural medium, sea water, the lowest possible permeability to water. This is interesting and rather surprising, though at present there is no evidence that the same holds true of cells in general.

The second method for testing the effect of the valence of the cation is to find the lowest concentration of each salt that will produce permeability as low as in sea water. Do these critical concentrations form a regular series corresponding to the valence of the cation?

In preliminary experiments with the series $ThCl_4$, $LaCl_3$, $CaCl_2$ and NaCl indications of a positive result were obtained. But solutions of $ThCl_4$ and $LaCl_3$ were highly acid and were therefore regarded as unsuitable. A cation series was then found which gave practically neutral solutions. This was a series of cobaltammine chlorides. The cations of these salts ranged in valence from 6 to zero (the last an undissociated compound). The following are representative of a larger number of salts actually used.

$$\left[\mathrm{Co}\begin{pmatrix}\mathrm{OH}\\ \mathrm{OH}\end{pmatrix}\mathrm{Co}(\mathrm{NH_3})_4\right]_3\mathrm{Cl_6}$$

$$\left[(\mathrm{NH_3})_4\,\mathrm{Co}\begin{matrix}\mathrm{O_2}\\ \mathrm{NH_2}\end{matrix}\mathrm{Co}(\mathrm{NH_3})_4\right]\mathrm{Cl_4}$$

$$[\mathrm{Co}(\mathrm{NH_3})_6]\,\mathrm{Cl_3}$$

$$\left[\mathrm{Co}\begin{matrix}\mathrm{Cl}\\ (\mathrm{NH_3})_5\end{matrix}\right]\mathrm{Cl_2}$$

$$\left[\mathrm{Co}\begin{matrix}\mathrm{Cl}\\ \mathrm{NO_2}\\ (\mathrm{NH_3})_4\end{matrix}\right]\mathrm{Cl}$$

$$\left[\mathrm{Co}\begin{matrix}(\mathrm{NO_2})_3\\ \\ (\mathrm{NH_3})_3\end{matrix}\right]$$

Such salts are reported to undergo practically complete electrolytic dissociation.

With this cation series, striking evidence of valence effect was obtained under the following conditions. To 0.38 molal dextrose was added a small amount of a salt which had been found to increase permeability, e.g., K_2SO_4 or K_3 citrate. Various amounts of a cobaltammine

were added and the lowest concentration found which gave a permeability value as low as that found in sea water. These critical concentrations formed a regular series corresponding to the valence of the cation.

A representative experiment follows.

MOLAR CONCENTRATION	2.5×10^{-5}	5×10^{-5}	1×10^{-4}	2×10^{-4}	4×10^{-4}	8×10^{-4}	1.6×10^{-3}	3.2×10^{-3}
6 valent	0.194	0.048	0.042					
4 valent		0.172	0.058	0.038				
3 valent			0.180	0.068	0.061			
2 valent					0.171	0.113	0.157	0.043
1 valent								0.058

Cobaltamnines were added to a solution consisting of 0.38 molal dextrose and 0.005 molal K_3 citrate. The table shows that 0.00005 M. of the 6 valent salt was sufficient to reduce permeability to the value found in sea water at the same temperature, more than twice as much of the 4 valent cobalt was required, more than 8 times as much 3 valent and 64 times as much of the 2 valent salt, while this amount of the 1 valent salt was incompletely effective.

Thus it is seen that there is a definite valence effect and that the effectiveness of a salt in reducing permeability increases rapidly with valence of its cation.

Equally decisive results were obtained with anion series. Two such were used: K_3 citrate, K_2 tartrate and K acetate; $K_4Fe(CN)_6$, $K_3Fe(CN)_6$, K_2SO_4 and KCl. These were added to a solution consisting of 0.38 molal dextrose and 0.0005 molal $CaCl_2$. Before addition of potassium salts this solution yielded the same permeability value as sea water. The lowest concentration of the various salts was determined which decisively increased permeability.

The accompanying table shows a representative experiment.

MOLAR CONCENTRATION	2×10^{-4}	8×10^{-4}	1×10^{-3}	2×10^{-3}	4×10^{-3}	8×10^{-3}
KCl				0.066	0.067	0.095
K_2SO_4				0.048	0.152	
$K_3Fe(CN)_6$			0.068	0.107		
$K_4Fe(CN)_6$	0.056	0.063	0.087	0.192		

($CaCl_2 = 0.046$)

0.001 M. ferrocyanide was required definitely to increase permeability, twice as much ferricyanide, 4 times as much sulfate and 8 times as much chloride.

Thus it was found that cations tend to decrease permeability and anions to increase it, and that in both cases the effectiveness of the ion increases rapidly with its valence. In this low concentration range evidently valence prevails over other ionic effects. It appears that antagonism is not between cations of different valence, e.g., K and Ca, but between cation and anion; that this antagonism is an expression of the sign of charge on the ion.

Such a conclusion, of course, does not deny that in higher concentrations antagonisms may be demonstrable between ions of the same sign.

c. Jacobs (55) working with erythrocytes measured the time required to produce a certain degree of hemolysis in distilled water and in solutions of certain electrolytes. These retarded hemolysis. The lowest concentrations in which a definite retarding effect was observed were as follows: NaCl, 0.001 M.; $CaCl_2$, 0.0001 M.; Al_2Cl_6, 0.00004 M. It is seen that the effect of these electrolytes increases with the valence of the cation.

It is interesting to note that valence and sign of the charge were found by Osterhout (97, 97 a) and Raber (104, 105, 106) to produce changes in conductivity of plant tissues that are closely paralleled by the experiments on *Arbacia* cells and erythrocytes just cited.

C. *Effect of activation (fertilization) of echinoderm eggs on permeability to water.* Among the many chemical and physical changes that occur in the egg cell on fertilization, its increased permeability to water-soluble and diffusible substances is regarded by Lillie (70) as of fundamental importance. Evidences of such permeability change, quoted by Lillie, are increased electrical conductivity, the readier entrance of substances like alkali and certain dyes, the loss of certain substances (e.g., pigment) from the fertilized egg, and the fact that pure isotonic solutions of sodium salts, which are known to increase permeability and to serve as activating agents, do neither in the presence of calcium or magnesium salts or anesthetics.

In this connection the observation of Loeb (79) is recalled, that parthenogenetic agents are in general cytolytic ones. This is true of the pure salt solutions and acids (e.g., butyric) ordinarily used to activate the egg artificially, as well as agents less often used, such as electric currents, heat and mechanical stimulation. Lillie sees a close connection between the activating effects of these agents and their stimula-

tion of irritable tissues. In the case of both activation and stimulation, he regards the first step as increase in permeability of the cell surface, as the result of which occurs a change in electrical polarization; electrical changes he regards as the critical phenomenon which brings about either activation or stimulation.

Since the permeability to water-soluble substances is higher in fertilized than in unfertilized eggs, Lillie was led to determine whether there was a similar difference in relation to water itself. When he placed both fertilized and unfertilized eggs in hypotonic sea water, the fertilized ones had after a few minutes swollen more than the others. By means of accurate measurements of the course of swelling, Lillie developed a sensitive quantitative measure of permeability differences. His mathematical treatment has been given above. By means of equation (1), (see section on kinetics) he found that the velocity constant of swelling was 4 times greater in the case of fertilized eggs, in other words, that these were 4 times as permeable to water as unfertilized eggs. This striking increase in permeability persists at least until the second or third cleavage, and probably longer (71).

Eggs in which fertilization membranes had been produced by treatment with butyric acid also showed an initial permeability increase of 4 fold, but in this case equation (1) fitted the data poorly, since K steadily declined, suggesting partial loss in semipermeability of the cell surface.

Thus Lillie concluded that fertilized eggs are several times more permeable to water than are unfertilized ones. However, this interpretation of his data was questioned by Jacques Loeb (78), who thought that the apparent increase in permeability might be due to loss of the jelly envelope. This is usually found enclosing unfertilized eggs, but is commonly lost on fertilization. Lillie (71), however, found that removal of the jelly from unfertilized eggs by washing does not result in increased permeability.[13] By this and other evidence he showed Loeb's objections to be invalid. Apparently the jelly is so dilute that it exerts no appreciable retarding effect on diffusion.

Lillie also pointed out that although water enters the fertilized egg more rapidly, yet the tendency to cytolysis in hypotonic solutions is much less than before fertilization. Fertilized eggs remain intact in 40 per cent sea water for several hours, while unfertilized ones are mostly broken down within an hour.

[13] In recent experiments Leitch (69) also found that the jelly enveloping the eggs of the sand-dollar, *Dendraster*, has no appreciable effect on their permeability to water.

In subsequent work (72, 73) Lillie developed another method for detecting changes in permeability, a method having the advantage of extreme simplicity. When a mixed suspension of unfertilized and fertilized eggs is placed in hypertonic sea water or van't Hoff's solution, having an osmotic pressure of 35 to 40 atmospheres, both kinds of eggs of course give up water and shrink. But shrinkage occurs at very different rates in the 2 cases. When observed through the microscope one minute after transfer to hypertonic solution, unfertilized eggs have not shrunk enough for the change in size to be obvious without measurement, but the fertilized eggs are already greatly shrunken and exhibit in addition a characteristic change in shape. The cytoplasm is no longer rounded but angular, crenated. This difference in appearance of the two cells is so striking as to afford a ready means of detecting permeability changes, though, owing to crenation of the fertilized eggs, measurement is not practicable.

Eggs in which a fertilization membrane has been artificially produced by treatment with butyric acid also shrink rapidly. But such eggs differ from both unfertilized (untreated) and naturally fertilized ones in that their permeability is quite variable. Some shrink as rapidly as fertilized eggs while others in the same microscopic field behave almost like unfertilized ones. Some cells treated with butyric acid have well formed membranes while in others the membrane is narrow and poorly developed; the former shrink rapidly in hypotonic sea water, the latter more slowly; also when the membrane is well formed the cell is likely to undergo crenation and to lose some pigment.

Permeability to water in Echinarachnius (sand-dollar) eggs. These behave much as do *Arbacia* eggs being similarly affected by fertilization. In a single experiment with star-fish eggs, differences between fertilized and unfertilized eggs were not evident, both swelling or shrinking rapidly in anisotonic solutions.

Returning to fertilized *Arbacia* eggs it was found that increase in permeability is not instantaneous. It begins probably 2 to 4 minutes after insemination and is in greater part completed during the next 10 minutes. This was demonstrated by transferring eggs to hypotonic sea-water at different intervals after insemination. During the first few minutes after sperm has been added, eggs in hypertonic solutions shrink slowly and remain round like unfertilized eggs. After 5 to 6 minutes from the time of insemination, noticeable increase in rate of shrinkage occurs, and this becomes more rapid at the same time that the tendency to crenation becomes more pronounced. Increased water permeability

appears to remain as a permanent quality of the developing egg and may readily be demonstrated in the 2-cell and 4-cell stages in intervals between cleavages.

Increased permeability continues long after the complete separation of the fertilization membrane, and cannot be directly referred to the formation of this structure or to any changes in its properties after separation, but is due rather to progressive changes in the osmotic properties of the semi-permeable surface layer of protoplasm. Lillie regards these changes as intimately connected with increased cellular metabolism, which expresses itself also in increased output of CO_2 and greater susceptibility to cyanide.

The correctness of this view is further evidenced by the effect of narcotics. If the increase in permeability brought about by fertilization were due to altered and increased cellular metabolism, it seemed likely that anesthetics and cyanides would prevent such a change. This proved to be the case.

Two or three minutes after insemination, eggs were placed in m/100 or m/200 KCN for half an hour. When examined subsequently in hypertonic sea-water, these cells shrank very slowly and only a few became crenated. A concentration of m/800 KCN was without effect, while in m/400 only a partial increase in permeability was noted; 0.2 per cent chloral hydrate also prevented the increase in permeability from occurring, as did appropriate concentrations of other narcotics (chloroform, methyl alcohol, ethyl alcohol, n-propyl alcohol, isobutyl alcohol, i-amyl alcohol, ethyl ether). Such effective concentrations were frequently higher than those sufficient to prevent cleavage.

This effect of narcotics was reversible, for when eggs were returned to ordinary sea water for 30 minutes, they subsequently shrank just as rapidly in hypertonic solutions as eggs that had not been treated with narcotics. These eggs went on to normal cleavage, showing that injury had not occurred.[14]

Thus it is seen that anesthetics and cyanides are able to prevent increase in permeability to water following insemination. The question then arose, are they also able to cause a return to low permeability after increase in permeability has occurred? The answer to this question varies with the agent employed. Ethyl urethane was the most effective. Eggs, 30 minutes after fertilization, were placed in sea water

[14] In contrast to the action of cyanide and anesthetics, calcium, in high concentration, did not prevent increased permeability to water, though in such a solution cleavage would not occur.

containing 2 per cent ethyl urethane, and 20 minutes later transferred to hypertonic sea water containing the same concentration of anesthetic. Shrinking was very slow and all eggs remained round. Chloral hydrate and the alcohols caused well marked decrease in permeability, but ether, chloroform and cyanide had little or no effect.

D. Narcotics. Most studies of the effect of narcotics on cell permeability have been concerned only with permeability to various substances in solution. There are, however, a number of investigations which deal with permeability to water as influenced by narcotics.

(a) Winterstein (128) in his first group of experiments used sartorius muscle of frogs, employing the usual method of weighing. Since cells are freely permeable to narcotics (as was shown by Overton) the addition of a narcotic to a solution does not change the osmotic pressure difference between the solution and the interior of the cell suspended in this medium.

If therefore two sartorius muscles are placed in different samples of the same anisotonic solution, one of which contains a narcotic, both muscles should show the same changes in weight, provided that the narcotic does not affect permeability. If, however, permeability is affected by the narcotic, a difference in weight would result during the swelling process. Winterstein found that in a hypotonic solution of NaCl containing 4 to 5 per cent of alcohol the weight increase of muscle was less than in the same hypotonic solution containing no narcotic. For example, a frog's sartorius transferred from 0.7 per cent to 0.35 per cent NaCl solution increased 32.3 per cent in weight in one hour's time; the other sartorius of the same frog gained only 22 per cent in the same time after transfer from 0.7 per cent NaCl + 4 per cent alcohol to 0.35 per cent NaCl + 4 per cent alcohol.

In later experiments Winterstein constructed artificial "cells;" the open ends of small glass cylinders were covered with the thin abdominal muscle of frogs. In the control experiments the cells were filled with 0.7 per cent NaCl and placed for a certain length of time in a hypotonic solution, generally distilled water. In the narcosis experiments a given amount of narcotic was added to both the solution inside the "cell" and the outside solution. The amount of water transferred was determined by weighing the "cells;" diffusion of salt from the "cell" was determined by analysis. With four different narcotics (chloroform, ether, ethyl urethane, alcohol) Winterstein found a definite decrease in permeability of the muscle membrane to water. A typical experiment may be cited as illustration.

	NARCOSIS EXPERIMENT "CELLS" FILLED WITH 0.7 PER CENT NaCl CONTAINING 6 VOLUMES PER CENT ALCOHOL. PLACED FOR 1 HOUR IN DISTILLED WATER CONTAINING 6 VOLUMES PER CENT OF ALCOHOL	CONTROL EXPERIMENT "CELLS" FILLED WITH 0.7 PER CENT NaCl AND PLACED FOR 1 HOUR IN DISTILLED WATER
	per cent	*per cent*
Water intake	1.4	7.2
Diffusion of salt	1.1	1.6
Final concentration of NaCl.	0.68	0.64

At the end of this experiment the muscle membranes of the "cells" were placed for 50 minutes in 0.7 per cent NaCl. Both cells were then filled with 0.7 per cent NaCl and placed in distilled water for 1 hour.

Water intake	8.1	8.3
Diffusion of salt	0.0	0.4
Final concentration of NaCl.	0.65	0.64

The experiments show the decrease of permeability to water, and the complete reversibility of the process. Winterstein further showed that subnarcotic amounts of alcohol had no effect on permeability, while toxic doses of the various narcotics produced an irreversible increase in permeability to water.

(b) The experiments of Winterstein were extended by Lillie (72, 73) to an investigation of the effects of narcotics on permeability to water of isolated cells, the fertilized eggs of *Arbacia*. This work has already been reviewed in connection with the effects of activation.

(c) Heilbrunn (43) repeated certain of Winterstein's experiments, studying the increase in weight of frog's gastrocnemius in distilled water, and in distilled water to which 2 per cent by volume of ether had been added. He found that water entered etherized muscle somewhat less rapidly than normal muscle. In further experiments Heilbrunn investigated the rapidity of swelling of unfertilized eggs of *Arbacia* in hypotonic sea water containing one or two per cent of ether. His curves show that the cells swelled even more readily in the presence of ether than in its absence. From his experiments Heilbrunn concluded that ether does not lower the permeability of sea urchin eggs to water. It is not improbable, however, that the more rapid swelling of the narcotized cells in Heilbrunn's experiments was due to injury.

(d) In the case of membranes there are two recent investigations con-

cerning the effect of narcotics on permeability to water. Czaja (12) used as his material the bladders (utricles) of several species of *Utricularia*. The weakly cutinized external membrane of the utricle is normally permeable to water but impermeable to dissolved substances. Various narcotics in low concentration led to reversible decrease in permeability of the membrane to water. Beyond a certain concentration, the compounds cause damage and irreversible increase in permeability. Anselmino (1) studied the effect of narcotics (urethanes and alcohol) on permeability to water of collodion and copper ferrocyanide membrane and found a marked reversible decrease in permeability. He regarded this decrease in permeability as due to mechanical diminution in the size of the pores of the membrane caused by adsorption of the narcotic on the walls of the pores.

(e) In the case of erythrocytes the effect of narcotics on permeability to water is somewhat difficult to evaluate with the methods of hemolysis that have been employed.

Arrhenius and Bubanovic (2), and Jarisch (60) investigated the effect of various narcotics upon hemolysis of erythrocytes in hypotonic solutions. They observed that narcotics which in high concentration increased hemolysis had, in low or narcotic concentrations, the opposite effect. The retardation or prevention of hypotonic hemolysis was interpreted as indicating a decrease in permeability to water. On the other hand, Yoshitomi (131) reported that only certain narcotics increased the resistance of erythrocytes to hypotonic solutions; other narcotics had no effect, or increased hemolysis. Jacobs and Parpart (58) found, with a concentration of ethyl urethane of 0.3 M. in a given hypotonic solution, the course of hemolysis to be scarcely less rapid than in its absence. The urethane had no effect on the time required to attain equilibrium. Measurement of the rate of hemolysis in pure water and in strongly hypotonic solutions also showed only a slight retardation by various urethanes and alcohols in low concentration. Jacobs (58) concludes that much caution is necessary in the interpretation of the experiments previously reported. He discusses the factors which may influence hemolysis of erythrocytes in hypotonic solutions and suggests that narcotics may cause not a decrease in permeability to water, but to hemoglobin; or, that narcotics may shift the equilibrium volume toward which the corpuscles tend to swell in hypotonic solutions.

(f) Recently Lucké (81) determined the effect of several urethanes and carbamates on permeability to water of unfertilized *Arbacia* eggs. These substances in narcotizing concentrations (as determined by fer-

tilization tests) were dissolved in hypotonic sea water. In these solutions the course of swelling was followed and permeability was calculated at a certain instant. Values thus obtained were compared with permeability in sea water without addition of the narcotic. Under these conditions no differences in permeability were found, i.e., these narcotics did not lower permeability below the value of the controls. However, high concentrations of urethanes and carbamates, e.g., 0.1 M. n-propyl urethane and 0.05 M. n-butyl carbamate, were toxic and increased permeability.

Previous work of McCutcheon and Lucké (89) had shown that in sea water and its dilutions the permeability of unfertilized *Arbacia* eggs is very low. This condition was found to be due to presence of Ca and Mg. These cations are so effective, even in concentrations as low as 0.0001 M., that permeability cannot be further lowered, e.g., by addition of multivalent cations.

It seemed possible, therefore, that in the experiments with urethanes and carbamates, permeability reducing properties of these narcotics had been masked by the presence of Ca and Mg.

Accordingly experiments were done in which cells were caused to swell in solutions containing no Ca and Mg; 0.38 M. dextrose solution was used, which is isotonic with 40 per cent sea water. To this solution the narcotics were added and the course of swelling followed.

It was found that permeability was considerably lowered by the presence of narcotics Thus in 0.38 M. dextrose without narcotics permeability varied between 0.092 and 0.097. With addition of 0.025 M. n-butyl carbamate, permeability was 0.062; with 0.01 M. i-amyl carbamate, 0.085; with 0.0025 M. phenyl urethane, 0.070. These values could not be lowered by increasing the concentrations of the narcotics. However, these narcotics were decidedly less effective in reducing permeability than was 0.01 M. $CaCl_2$. This concentration of $CaCl_2$ in 0.38 M. dextrose gave values ranging from 0.041 to 0.047, which are the same as values obtained with hypotonic sea water at the same temperature (12°C.). It is seen that with the narcotics permeability is about midway between the value obtained with dextrose alone and with dextrose plus $CaCl_2$.

It appears, then, that the permeability reducing property of narcotics may be masked under certain conditions. Apparently unfertilized *Arbacia* eggs in sea water have already so low a permeability that it cannot be further reduced. But the case is different in cells whose permeability to water is higher: narcotics then are able to reduce perme-

ability. This may be demonstrated with *Arbacia* eggs by increasing their permeability in some way, e.g., by fertilizing them, as in the experiments of Lillie (72), or by removing bivalent cations from the solution, as in the experiments just cited.

In general, the effect of narcotics on permeability to water appears to be as stated by Winterstein (129), namely, narcotics in those concentrations which produce reversible inhibition of function also cause reversible decrease in permeability to water (as well as to water soluble substances). In toxic concentrations, however, narcotics lead to cell damage and consequent increase in permeability.

E. The effect of injury on cellular permeability to water. There are few types of experiments with living cells in which the question does not arise, has the material been injured by the experimental procedure and are the results obtained dependent in part or whole on injury? It is, therefore, highly desirable that one should be able to apply to the material precise criteria of injury, and that by intentionally inflicted injuries of various types one should determine for each type the effect on the cell property which is being investigated.

Marine ova possess the advantage of offering a definite criterion of injury: at the end of an experiment when the cell is replaced in its normal environment, i.e., ordinary sea water, and inseminated, uninjured cells undergo normal cleavage and development, whereas injured cells divide atypically or not at all.

(a) The effect of 2 types of injury on permeability to water of unfertilized sea urchin eggs has been investigated by Lucké and McCutcheon (85). These types are high temperature and low osmotic pressure of the medium. In each case it was shown that under suitable conditions, permeability to water is increased.

The effect of heat was determined, first, by exposing cells for a definite time to different degrees of heat or, second, by heating cells at a definite temperature for varying lengths of time. Afterwards the cells were cooled and then caused to swell in hypotonic solutions.

There are of course limits within which such an experiment can be made successfully. If cells are heated at a temperature insufficiently high or for too short a time, the cells behave normally, that is, they are "uninjured" in the sense here employed. At the other limit of injury cells are firmly coagulated and do not swell at all.

The first type of experiment dealt with the effects of different dilutions of sea water on previously heated cells. Such an experiment is illustrated in the accompanying graph (fig. 5), which may be contrasted

with figure 2, in which is represented the course of swelling of previously normal cells. The curves in the 2 graphs are seen to be quite different. Thus normal cells placed in 20 per cent sea water swell rapidly until, having passed their limit of elastic resistance, they burst. Heated cells, however, at first swell very rapidly indeed, but then decrease in volume. In a less striking way, heated cells exposed to 60 per cent sea water follow a similar course of swelling and shrinking, whereas the swelling curve in 80 per cent sea water is, qualitatively at least, more nearly normal.

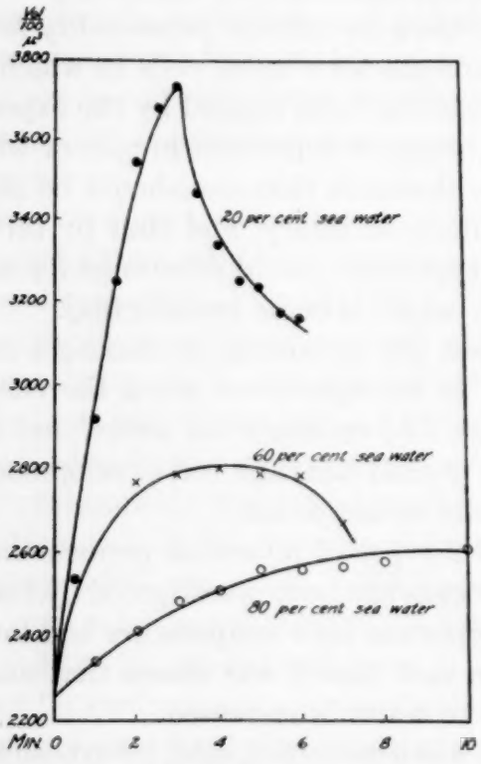

Fig. 5

The explanation of the abnormal curves is believed to be as follows. Injury of the cell is associated with more or less loss of the property of semipermeability, that is, the cell becomes permeable to substances which normally are able to cross its surface with difficulty or not at all. This change in the properties of the cell surface allows salts and even pigment and other cell contents to escape. At the same time, permeability to water is increased. However, loss of semipermeability is not complete, at least immediately. The cell, injured under the conditions of these experiments, when placed in hypotonic solutions is still more

permeable to water than to cell contents, so that at first it swells with great rapidity. With increasing distention of the cell, permeability to cell contents increases until their loss overbalances endosmosis of water. There follows, then, the decrease in cell volume shown in the abnormal curves.

It should be pointed out that the amount of injury suffered by cells under given conditions is unequal. Some cells are altered more, some less. The degree of injury sustained varies with the previous history of the cell and probably with various other factors, so that even in cells from the same animal, treated in precisely the same way, the course of swelling and of shrinking varies appreciably. In other words, injured cells are more variable in behavior than are normal cells.

The next group of experiments was designed to answer the question, are there quantitative differences in permeability to water between normal and heated cells? Cells were heated at 39°C. for times varying from 2 to 16 minutes. They were then cooled and caused to swell in 40 per cent sea water at 20°C. Permeability computed at the first minute of the swelling process, as described above, was in the case of unheated (control) cells, 0.069 cubic micron per minute, per square micron of cell surface per atmosphere of pressure. Heated cells yielded the following values: heated for 2 minutes, 0.076; 4 minutes, 0.081; 8 minutes, 0.084; 12 minutes, 0.119; 16 minutes, 0.172. Thus a regular series of values was obtained which varied with the length of exposure. Fertilization tests showed atypical division of ova heated for 2 minutes, and no cleavage after longer exposure.

Similar results were obtained by varying the temperature for constant duration of exposure (4 minutes). Progressive increase in permeability was found in cells previously heated between 36.5° and 41°C. while at 45° cells were coagulated and therefore unable to swell in hypotonic solutions.

Injury by hypotonic solutions. Eggs of *Arbacia punctulata* may be exposed to considerable degrees of hypotonicity for hours without injury provided that the temperature is sufficiently low. Of course, the lower the osmotic pressure of the solution, the shorter the length of exposure required to produce injury Using the fertilization test as a criterion of injury it was consistently found that an increase in permeability to water accompanied such injury. In the accompanying table permeability at successive minutes is recorded for cells exposed to 3 different dilutions of sea water.

INTERVALS AT WHICH PERMEABILITY WAS CALCULATED	PERMEABILITY IN VARIOUS CONCENTRATIONS OF SEA WATER		
	30 per cent	40 per cent	50 per cent
minutes			
2	0.086	0.069	0.069
3	0.089	0.068	0.069
4	0.092	0.064	0.062
5	0.098	0.063	0.064
6	0.103	0.063	0.060
8	0.117	0.076	0.058
10		0.097	0.056
Cleavage	All cytolyzed	50 % divided; of these one-half atypical	All divided; 90 % typical

Values of k have been computed from the equation

$$k = \frac{dV}{dt} / S(P - P_{ex})$$

As previously stated, permeability thus calculated for normal cells either shows no drift or declines slightly. In the present experiment, cells in 50 per cent sea water follow such a course of swelling and are shown by fertilization not to have been injured. In 40 per cent sea water, permeability is seen to increase after the 6th minute and the fertilization test indicated injury, while in 30 per cent sea water permeability was high from the outset (this is unusual) and subsequently increased until cytolysis occurred.

Also in exosmosis of water close parallelism was demonstrated between abnormally high permeability and injury. The following is a representative experiment. Cells were swollen in various dilutions of sea water in which they remained for 4½ hours. They were then returned to ordinary sea water, the course of shrinking observed and permeability computed at the third minute. This value was 0.150 for cells previously swollen in the most dilute solution (50 per cent); only one-third of these cells remained capable of cleavage. Permeability values for the other dilutions were normal, viz.: 55 per cent, 0.083; 60 per cent, 0.089; 65 per cent, 0.093; 70 per cent, 0.094. In all of these from 90 to 95 per cent of cells subsequently divided normally.

It has been seen that injury by heat and by hypotonic solutions is attended by increased permeability of the cell to water. This factor—injury—must be taken into account in interpreting the effect of any

environmental factor on cellular permeability to water. It has been shown by other workers that injury is attended by increased permeability to various substances within and outside of the cell. Such increase in permeability, attended by decrease in the semipermeability of the cell surface, is one of the most important manifestations of injury, since loss of semipermeability is incompatible with the life of the cell.

(b) An important study of the effect of injury on permeability to water is Winterstein's (128). In his investigation he used artificial muscle "cells" similar to those employed by him in his studies on narcosis (v.s.). With this material he was able to differentiate clearly between change in permeability to water and change in permeability to salts. Winterstein found that cell damage leads not only to increased permeability to salts but to relatively greater increase in permeability to water. Thus in the following experiment an artificial "cell" was filled with 0.7 per cent NaCl solution and placed for one hour in distilled water. At the end of this time the "cell" was refilled with 0.7 per cent NaCl, and again placed in distilled water; the experiment was repeated at the end of the second hour. In each of these three successive experiments the amount of water diffusing into the artificial cell was determined by weighing, and the amount of salts diffusing out of the "cell," by analysis. The figures express per cent change.

	FIRST HOUR	SECOND HOUR	THIRD HOUR
Water intake	8.0	11.9	8.9
Diffusion of salt	4.3	5.4	8.8
Final concentration of salt in cell	0.62	0.59	0.52

This experiment shows that the increase of permeability of the muscle membrane to water is accompanied by increase in permeability to salt due, no doubt, to gradual injury of the cells. Although at the beginning of each experiment the difference in osmotic pressure between the interior of the "cell" and the medium is the same (i.e., 0.7 per cent NaCl against distilled water), there is seen to occur progressive leakage of salts, and even more marked increase in permeability to water. The absolute amount of water diffusing into the cell is increased in the second experiment. In the third experiment, in which the difference in osmotic pressure becomes diminished due to great leakage of salt, the increase in permeability to water is relatively even more marked.

(c) The effect of injury on permeability of the capillary wall to water was studied by Landis (68). He reviewed the evidence that, following

injury of the capillary wall, permeability is increased to such an extent as to permit escape of the plasma colloids (67), and Landis himself showed that with injury capillaries become quite permeable to semi-colloidal dyes as well (68). It was to be expected, therefore, that injured capillaries would have an increased permeability for fluid. Landis devised an ingenious micro-injection method by means of which he was able to determine with accuracy the rate of passage of fluid through the capillary wall, as well as the intracapillary pressure. He perfused the mesenteric capillaries of frogs with 10 per cent alcohol in Ringer's fluid, and found that filtration permeability was increased approximately seven times above the value determined for normal capillaries. In other experiments he injured the capillary endothelium with 1:10,000 mercuric chloride solution and obtained essentially the same degree of increase in permeability to fluid.

(d) The effect of injury produced by narcotics on permeability to water is of particular interest, for, as is pointed out elsewhere, narcotics in suitable concentration tend to decrease permeability to water as well as to substances in solution. In toxic concentration, however, narcotics have the opposite effect, bringing about increased permeability to water (as well as to dissolved substances). In our own experiments with sea urchin eggs (81) it was found that when the narcotic caused injury to the cells, as determined by failure of cleavage, the values for permeability were increased. Thus in a group of experiments in which different concentrations of certain urethanes and carbamates were caused to act in sea water it was found that toxic doses effected an increase in permeability of from 75 to 100 per cent above the normal.

Winterstein had similar results using the artificial muscle "cells" already described (128). Thus he found that while 0.12 vol. per cent of chloroform effected a decisive reversible decrease in permeability to water, 0.3 vol. per cent caused irreversible increase in permeability. He further showed that *prolonged* exposure to narcotic concentrations (for example, 0.12 per cent chloroform) brings about injury and irreversible increase in permeability.

(e) Finally it should be pointed out that the cell when it dies loses the properties of an osmotic system. Its semipermeability is destroyed. It may swell rapidly in hypotonic, in isotonic and even in hypertonic solutions, apparently due to increase in osmotic pressure of the cell contents. But because of progressive loss of semipermeability, cell contents escape, and swelling may be followed by shrinkage.

Under these circumstances Avogadro's law (and the other simple

laws of osmosis) no longer apply. Volumes of dead cells may be quite different in solutions of the same osmotic pressure. Like gelatin, the volume of dead cells depends largely on the chemical composition of the solution rather than essentially on osmotic pressure.

Loeb (75, 80) was probably the first to point out that although muscle maintains constant weight in all non-toxic isotonic solutions for a certain time, it gains weight when death occurs. Similar observations have been made by Webster (126), Fletcher (29), Beutner (6, 7), Winterstein (127), von Körösy (64) and others.

Loeb (76) also showed that muscle while it remains alive and uninjured loses water in hypertonic solution; after a certain length of time, however, the muscle becomes injured and begins steadily to increase in weight.

With isolated cells (*Arbacia* eggs), Lucké and McCutcheon (82) have studied the course of the swelling of dead cells in various isotonic solutions. Using diverse injurious agents, such as acids, ether and heat, it was found that the cells became coagulated and rapidly increased in size. It was repeatedly observed that the magnitude as well as the rapidity of swelling depended on concentration or amount of the injurious agent. Thus 0.005 molar salicylic acid in ordinary sea water caused very rapid swelling with great volume increase, while 0.0025 salicylic acid effected a much slower and less marked swelling. When observations were extended over a sufficient length of time it was generally observed that the swelling was followed by volume decrease.

The experiments reviewed in this section show the necessity of carefully considering the possibility of injury or death in osmotic experiments with cells and tissues.

F. *Comparative permeability to water of various cells and membranes.* It may be of interest to compare the values for permeability to water determined for several kinds of living cells, and for living and non-living membranes. These values represent the number of cubic micra of water passing through one square micron of surface per minute per atmosphere of pressure, at 20°C.

The permeability of the unfertilized egg of the sea-urchin, *Arbacia*, is about 0.1 (86). In the fertilized *Arbacia* egg permeability is increased two to four times (73). The unfertilized eggs of several marine invertebrates from the Pacific Coast have a somewhat higher permeability though the values are of the same order of magnitude (69).

On the other hand, erythrocytes are many times more permeable to water than marine egg cells, the permeability for human red corpuscles being of the order of magnitude of 3.0 (58).

In membranes of living systems an extreme range of permeability is found, from practically complete impermeability to free permeability. Thus, under certain conditions, the membrane of the egg of *Fundulus* is practically impermeable to water in a physiologically balanced solution (79). The same is true of certain plant cells with cutinized or suberized walls (113). The other extreme is found in the cellulose wall of many plant cells which permit free diffusion of water.

The permeability to water of the capillary wall is very great. In the present units the value for mesenteric capillaries of the frog is about 370 (68), or over one hundred times as great as the permeability of erythrocytes, and almost four thousand times as great as the permeability of marine eggs.

Non-living membranes may be prepared of almost any desired permeability. Northrop (96) has even succeeded in preparing thin dry collodion membranes the permeability of which is of the same order of magnitude as is the case in the *Arbacia* egg.

It should be pointed out that *comparative* values of permeability of different cells and membranes, though of interest, are of doubtful significance inasmuch as the thickness of cell membranes is extremely difficult to estimate with any degree of exactitude.

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